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(54) Title: BLOOD-BRAIN BARRIER MODEL (57) Abstract <p>An <i>in vitro</i> model of a blood-brain barrier comprising a porous solid support upon which is disposed an essentially confluent monolayer of brain microvascular endothelial cells in contact with agents that elevate effective cyclic AMP concentrations in endothelial cells, with or without astrocyte-derived or endothelial cell-derived conditioned medium or the equivalent so that high electrical resistance tight junctions are formed between endothelial cells, and endothelial cells exhibit peripheral phalloidin staining and E-cadherin. Also disclosed is the use of agents that reduce effective cyclic AMP concentrations or interfere with the functioning of cyclic AMP or increase effective cyclic GMP concentrations to open up blood-brain barriers <i>in vitro</i> and <i>in vivo</i>, so that drugs normally excluded by such barriers may substantially penetrate such barriers. Also disclosed are uses of the model to screen for reagents with clinical utility in disorders involving brain endothelial cells.</p>		

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BLOOD-BRAIN BARRIER MODEL

This is a continuation-in-part of United States Serial No. 097/413,274, filed September 27, 1989.

BACKGROUND OF THE INVENTIONField of the Invention

5 The present invention relates generally to in vitro models of endothelial cells. More particularly, the invention relates to an in vitro model that simulates the characteristics of microvascular endothelial cells of the brain that constitute the
10 blood-brain barrier.

Description of Related Art

The vertebrate brain has a unique capillary system unlike that of any other organ of the body. This unique capillary system has morphological and
15 biochemical characteristics that make up the "blood-brain barrier" (BBB). The BBB acts to separate the brain interstitial space from the blood. This barrier prevents molecules in the blood that are neither lipophilic or transported by specific carrier proteins
20 from entering the brain (Betz, A.L. et al., Ann. Rev. Physiol., 48:241 (1986); Pardridge, W.M., Ann. Rev. Pharmacol. Toxicol., 28:25 (1988)).

The characteristics of the brain capillaries that make up the BBB include: (a) high-resistance tight
25 junctions between endothelial cells of the brain that block transport of molecules between cells; and (b) limited amount of transport across cells, as compared to that occurring in peripheral capillaries.

0 The tight junctions of the BBB prevent passive
diffusion of molecules and ions around the endothelial
cells. Thus, most hydrophilic drugs and peptides that
gain ready access to other tissues of the body are
barred from entry into the brain, or their rates of
5 entry are low. Thus, at the BBB, the only substances
that can readily pass from the luminal core of the
capillary to the abluminal tissue that surround the
capillary are those molecules for which selective
transport systems exist in the endothelial cells, as
10 well as compounds that are lipid soluble. Such
compounds, because of their inherent lipophilicity, are
able to intercalate into the plasma membrane of
endothelial cells and move to the abluminal side.
These unique properties of the BBB have provided a
15 major hindrance to the development of therapeutic
agents directed toward diseases of the central nervous
system (CNS), e.g., Alzheimer's disease and Parkinson's
disease.

 There are two general situations in which the
20 ability to test for CNS entry of therapeutic agents is
important. First, the increasing prevalence of CNS
disorders and the introduction of new molecular
biological and biochemical techniques to treat such
disorders will lead to the development of new drugs
25 that will be centrally active. These drugs must be
tested for their ability to reach the brain, i.e.,
penetrate the BBB. Second, many drugs used to treat
peripheral disorders have undesirable CNS side effects.
As replacements for these drugs are developed, they
30 will have to be screened for CNS penetration as well.
Of course, the objective in that case is to develop
peripherally-acting drugs that do not enter the brain.

0 Screening batteries of compounds for passage into
the brain by conventional techniques is impractical.
Generally, compounds are introduced into the carotid
artery, and their concentration in the brain is then
determined. This means that for each individual
5 compound many animals must be injected and processed.
While animal testing in vivo is important, it is not
the optimal screening system when many compounds have
to be examined.

Thus, it would be highly desirable to have an in
10 vitro model of the BBB so as to be able efficiently and
inexpensively to screen numerous drugs in a relatively
short amount of time. The test system should closely
simulate the morphological and physiological
characteristics of the in vivo BBB in having tight
15 junctions between cells and similar permeability
characteristics, and should be composed of defined cell
types.

Another desirable characteristic of an in vitro
model is that it should provide a system for testing
20 manipulations of the endothelial cells of a nature as
to increase or decrease the passage of drugs from the
blood side to the brain side of these cells.

Previous attempts to construct an in vitro model
of the BBB have not met the criteria outlined above.
25 Intact brain microvessels (Kumagai, A.K., J. Biol.
Chem., 262:15214 (1987)) are likely to contain not only
endothelial cells and astrocytes, but mast cells as
well. Further, the limited volume and access to the
lumen of microvessels precludes their use for vectorial
30 transport studies, and therefore makes them suboptimal
as a workable model for the BBB.

Several laboratories claim to have created a BBB
in vitro model using brain capillary endothelial cells

0 in the presence of standard growth media (Audus, K.L.,
et al., Ann. N.Y. Acad. Sci., 507:9 (1987); Van Bree,
J.B.B.H., et al., Pharm. Res., 5:369 (1988); Hart,
M.N., et al., J. Neuropath. Exp. Neurol., 46:141
5 (1987)). Cloned bovine brain capillary endothelial
cells, grown on a permeable support of glutaraldehyde-
treated collagen gel, have been reported to exhibit
high transendothelial cell resistance (Rutten, M.J.
et al., Brain Res., 425:301 (1987)). However, these
10 studies have demonstrated only one or a few of the
inherent morphological, biochemical and functional
characteristics of brain capillaries, and the data
derived from such systems are often conflicting, in
part because in most studies the systems employed
incompletely characterized populations of primary cell
15 cultures or cell lines, and in part because the brain
capillary endothelial cells were not grown in the
proper milieu.

It is known that brain astrocytes influence the
properties of brain capillary endothelial cells.
20 Janzer et al. (Janzer, R.C., Nature, 325:253 (1987))
disclosed that neonatal rat brain type 1 astrocytes,
cultured on filters and transplanted into the eyes of
syngeneic animals or chick embryo chorioallantoic
membranes, became vascularized by the endogenous
25 endothelial cells, and caused the endothelial cells to
exclude the dye, Evans blue.

Exclusion of Evans blue dye or other cationic dyes
that bind to albumin is one property of endothelial
cells in the brain. These results might be used to
30 predict that astrocytes can cause endothelial cells to
exhibit a generally low rate of macromolecular
transport. They do not necessarily indicate, however,
that the endothelial cells have been induced to form

0 the high resistance tight junctions which are also
characteristic of those cells in vivo.

Other in vitro studies have examined the effects
of brain astrocytes on ultra-structural properties of
endothelial cells. Brain astrocytes enhanced the
5 frequency, length and complexity of tight junctions
formed between cultured, brain-derived endothelial
cells (Tao-Cheng, J.-H. et al., J. Neurosci., 7:3293
(1987)). Also, fourth passage rat brain capillary
endothelial cell cultures, grown in rat brain
10 astrocyte-conditioned medium on endothelial cell
matrix-coated substrate, exhibited tight junction
biogenesis (Arthur, F.E. et al., Dev. Brain Research,
36:155-9 (1987)). Both studies relied solely upon
ultrastructural examination of individual groups of
15 treated cells, but neglected to look at resistance of
tight junctions.

Thus, an important need still exists for an in
vitro model of a BBB that meets all of the criteria
necessary for a model to simulate the in vivo
20 situation: 1) a monolayer of endothelial cells
essentially all of which are connected by tight
junctions; 2) a diffusion barrier for components that
do not ordinarily cross the BBB; and 3) a high
transendothelial cell electrical resistance barrier
25 indicating the presence of tight junctions that prevent
passive diffusion of ions.

SUMMARY OF THE INVENTION

In accordance with the present invention, an in
vitro model of the vertebrate BBB is disclosed that
30 simulates important morphological and permeability
characteristics of the brain BBB, that permits the.

0 efficient and inexpensive screening of CNS drugs, and
that allows testing of manipulations of the BBB.

The present invention is based on the effects of
the brain microenvironment on the special properties of
brain capillary endothelial cells. More specifically,
5 the present invention is based in part upon a
reconstruction of interactions between brain astrocytes
and brain capillary endothelial cells in in vitro
monolayer systems.

This invention is also based on the discovery that
10 treatments that raise effective cyclic AMP
concentrations in cultured brain endothelial cells,
particularly in conjunction with the presence of
components of endothelial cell and brain astrocyte-
derived conditioned media or the equivalent, markedly
15 increase the production of tight junctions that exhibit
properties of the BBB in vivo such as high electrical
resistance, peripheral staining of cells by phalloidin,
and a diffusion barrier for substances known ordinarily
not to cross the BBB.

20 This invention is also based upon the discovery
that tight junctions between brain microvascular
endothelial cells may be disrupted and the blood-brain
barrier made more permeable by agents that decrease the
effective intracellular concentration of cyclic AMP,
25 interfere with the physiological actions of cyclic AMP,
increase the effective intracellular concentration of
cyclic GMP, or promote the physiological actions of
cyclic GMP, and that such manipulations allow for the
facile delivery of drugs across the blood-brain
30 barrier.

It is thus an object of this invention to disclose
an in vitro model of the BBB comprising a porous solid
support separating monolayer cocultures of

0 microvascular endothelial cells and brain astrocytes
juxtaposed in a device that permits physiological
interaction between the cell types.

It is another object of this invention to disclose
an in vitro model of the BBB comprising a monolayer of
5 microvascular endothelial cells disposed on a filter in
contact with conditioned growth media derived from
endothelial cells or astrocytes.

It is yet another object of this invention to
provide criteria for selecting endothelial cells and
10 astrocytes particularly suitable for the in vitro
model.

It is a further object of this invention to
provide criteria for selecting substrata for the
culture of monolayers of cells in the in vitro model of
15 the invention.

It is still another object of this invention to
provide means for elevating the effective intracellular
cyclic AMP concentration in microvascular endothelial
cells of the in vitro model.

20 It is yet another object of this invention to
provide testing criteria for the genesis of tight
junctions in the in vitro model of the invention.

It is yet another object of this invention to
provide an in vitro model of a BBB which uses
25 endothelial cells from blood vessels other than brain
capillaries.

It is still another object of this invention to
provide compositions and methods involving
manipulations of cyclic AMP and cyclic GMP levels or
30 physiological effectiveness in order to open up blood-
brain barriers in vivo and in vitro and thereby to
permit drug delivery across such barriers.

0 These and other objects of this invention will
become clear by reference to the following disclosure
and appended claims.

DESCRIPTION OF THE FIGURES

- 5 Fig. 1 provides transmonolayer electrical
resistance data for the BBB model of the invention
using bovine brain capillary endothelial cell cultures.
Fig. 2 provides albumin flux data for monolayer
cultures of bovine retinal endothelial cells and MDCK
epithelial cells.
- 10 Fig. 3 shows flux data for sucrose and
chlorambucil across tight junction brain endothelial
cells.
- Fig. 4 shows the effect of agents that lower
cyclic AMP concentrations on morphine analgesia in
15 animals.
- Fig. 5 shows the effects of elevating endothelial
cell levels of cyclic GMP on cyclic AMP-induced tight
junctions.
- 20 Fig. 6 shows the effects of elevating in vivo
levels of cyclic GMP on morphine analgesia.
- Fig. 7 shows the effects of different classes of
cyclic AMP phosphodiesterase inhibitors on tight
junctions of brain endothelial cells in the BBB in
vitro model.

0 Fig. 8 Panels A and B, are photomicrographs of
sections from a brain sections in which MS-type
inflammation was induced via intracranial injection of
human tumor cells. Human and mouse lymphocytes were
allowed to contact the sections, and, as seen in Panel
5 A, bind selectively to exposed brain endothelium. In
Panel B, the lymphocytes were treated with an antibody
that inhibits the human VLA-4 receptor (anti-human β -1
integrin) and as can be seen, the human lymphocyte
(large cell) binding is substantially inhibited.

10 Fig. 9 is a photograph showing lymphocyte binding
and inhibition to binding in brain endothelial cells in
culture. Panel A shows the low level binding of
lymphocytes to the BBB model endothelium. In Panel
B, the endothelium has been treated with an inflammatory
15 reagent, and lymphocyte binding is increased
substantially. In Panel C, the lymphocytes were
pretreated with anti-human β -1 integrin monoclonal
antibody, and their binding to the stimulated
endothelium is substantially inhibited.

20 Fig. 10 is a graph showing the relative degree of
lymphocyte binding to blood vessels in sections of
inflamed brain tissue, and, as a basis for comparison,
normal lymph node tissue. The "no additions" column
shows brain tissue (scored) and lymph node endothelium
25 (solid) to which untreated lymphocytes have been added.
The degree of binding is represented as 100%. In the
next two columns, lymphocytes have been pretreated with
anti-VLA-4 reagents. The middle bars show lymphocytes
pretreated with anti- β -1 monoclonal antibody, the
30 right-hand bars show lymphocytes pretreated with anti-
 α -4 monoclonal antibody. In both cases, lymphocyte
binding in brain tissue is almost completely inhibited,
as compared to the control. But, lymphocyte binding to

0 lymph node endothelium, in both cases, is not significantly inhibited.

Fig. 11 is a graph showing the relative degree of Jurkat T-cell lymphocyte binding to brain endothelial cells in the BBB system. As can easily be seen, the anti- β -1 antibody effectively inhibited the binding of leukocytes to TNF- α activated brain endothelial cells. Anti- β -2, as a control, on the other hand, approaches the untreated control. Plainly, the β -1 subunit provides an effective target for preventing VLA-4/VCAM-1 interaction in the brain.

DETAILED DESCRIPTION OF THE INVENTION

The present invention comprises an in vitro model of mixed or cloned endothelial cells expressing high electrical resistance tight junctions and other properties of the BBB in vivo.

One embodiment of the invention comprises a chamber separated into at least two compartments by a porous solid support, on one surface of which support is disposed an essentially confluent monolayer of brain microvascular mixed or cloned endothelial cells growing on a particular substratum, the second compartment of the chamber housing an essentially confluent monolayer of brain astrocytes disposed either on a second surface of the chamber or on the underside of the porous solid support, the monolayers of both cell types being in sufficiently close juxtaposition so that products of each cell type can readily reach the cells of the other cell type. By "porous" we mean containing interstices through which water and the solutes contained therein, but not cells, can pass. In an alternative embodiment, the growth medium in contact with the endothelial cells contains, in part, an astrocyte or endothelial cell-

0 derived conditioned medium or equivalent. By
"conditioned medium" we mean a tissue culture growth
medium into which cultured cells have secreted
materials of cellular origin. By "equivalent" we mean
5 cell or tissue extracts containing materials of
cellular origin that may in other circumstances be
secreted extracellularly. Examples of the preparation
of conditioned mediums and equivalents are provided
below. In these embodiments of the model,
transcellular electrical resistance can be measured
10 directly, as described below. Details as to the
construction of these models will be provided below.

In another embodiment of the invention, mixed or
cloned microvascular endothelial cells may also be
grown on coated microcarrier beads, e.g., Cytodex-3
15 microcarriers (Pharmacia, Uppsala, Sweden), according
to Ryan et al. (Ryan, J., et al., Tissue Cell, 12:619
(1980)). Although in this model transcellular
resistance cannot be measured directly, macromolecular
transcellular transport, e.g., of labeled albumin,
20 cationized albumin, or glycosylated albumin, and of
dyes such as Trypan blue or Evans blue, can be
determined (Kempinski, O., et al., Acta Neuropathol.,
74:329 (1987); Bioadjieva, S., et al., Lab Invest.,
50:239 (1984); Smith, R.K., et al., Pharm. Res., 5:466
25 (1988)). The influence of astrocytes on endothelial
cells grown on microcarriers can be determined by first
growing brain astrocytes, such as neonatal rat type 1
astrocytes, on the beads, then removing the astrocytes,
leaving their extracellular matrix behind. This can be
30 accomplished either by lysing the astrocytes in 5 mM
Tris buffer, pH 7.4, containing 1% Triton X-100 for 15
minutes or by incubating the astrocytes in phosphate-
buffered saline (PBS) containing 10 mM EDTA for 30

0 minutes, both solutions containing protease inhibitors,
e.g., aprotinin and phenylmethysulfonyl fluoride.
Coated beads can be washed 3 times in PBS, then treated
with 25 mM NH_4OH . After the beads are washed again
with PBS, they can be coated with an essentially
5 confluent layer of cultured endothelial cells. Once
the endothelial cells reach confluence, they can be
maintained in a growth medium containing cultured
astrocytes, or in astrocyte-derived or endothelial
cell-derived conditioned medium.
10 In another embodiment, mixed or cloned
microvascular endothelial cells can be grown on porous
tube-like structures, such as those used in hollow-
fiber cell growth devices (Amicon Corp., Danvers, MA).
Again, a surface of hollow fibers can be coated with
15 astrocytes, from which astrocyte extracellular matrix
(ECM) can be prepared as described above. Endothelial
cells can then be grown on the astrocyte ECM, and the
cells exposed to astrocyte or endothelial cell-derived
conditioned medium. In this embodiment, transcellular
20 electrical resistance can be measured by passing
current between electrodes inside and outside the
hollow fiber. Macromolecular flux can be measured by
adding labeled macromolecules outside the fiber, and
following their transport across the endothelial cells
25 into the fiber.

Origin of Astrocytes

Purified populations of neonatal rodent brain
type 1 astrocytes were prepared according to the
procedures of Lillien et al. (Lillien, L.E., et al.,
30 Neuron, 1:485 (1988)). In brief, cerebral cortices
were removed from neonatal rats, white matter was
discarded, and the gray mater mechanically and

0 enzymatically (trypsinization) dissociated. Cells were
plated in poly-lysine-coated flasks in Dulbecco's
Modified Eagle's Medium (DMEM) plus 10% fetal calf
serum (FCS). After 5 days, loosely attached cells were
5 dislodged by shaking, attached cells were passaged into
new flasks, and treated with cytosine arabinoside (an
anti-mitotic drug) to remove actively proliferating
contaminating cells. Finally, astrocytes were
maintained in a chemically defined medium and fed twice
10 weekly. Cell type was determined by reactivity with
particular sets of antibodies. For example, type 1
astrocytes are fluorescently labeled by an antibody
against glial fibrillary acidic protein, but not with
the monoclonal antibody A2B5 (which labels type 2
astrocytes) or with an anti-galactocerebroside antibody
15 (which labels oligodendrocytes) (Raff, M.C., et al.,
J. Neurosci., 3:1289 (1983)).

Origin of Capillary Endothelial Cells

Endothelial cells are prepared from a variety of
animal and human sources. For example, mixed
20 populations of endothelial cells may be prepared from
purified capillaries derived from rodent and bovine
brain, bovine retina, bovine adrenal, bovine aorta, and
human omentum or from human umbilical vein. Bovine
sources are particularly suitable because of the large
25 amounts of tissue available, the ready availability of
fresh tissues, and the similarity of the permeability
of bovine capillary cells to that of their human
counterparts.

Bovine brain microvascular cells were isolated
30 according to Audus et al., Pharm. Res., 3:51 (1986)).
Briefly, a slurry of brain grey matter in Liebovitz'
L-15 medium was homogenized, and the microvascular

0 cell-containing particular fraction was separated on
a Dextran cushion. Capillaries were resuspended and
homogenized, then passed through a series of nylon
filters. Capillaries were digested further with
collagenase plus trypsin to provide a population of
5 single mixed endothelial cells. These cells were
plated on a collagen or fibronectin treated substratum
in 10% plasma-derived horse serum (PDHS) in Dulbecco's
modified Eagle's Medium (DMEM). Rat brain
microvascular endothelial cells were prepared similarly
10 according to Bowman, et al. (Bowman, P.D., et al., In
Vitro, 17:353 (1981)). Briefly, brain grey matter is
minced and digested with collagenase and dispase. The
particulate matter is separated over a 25% bovine serum
albumin (BSA) cushion, and the pellet further digested
15 with collagenase and DNase. Finally, endothelial cells
are isolated on a Percoll gradient, and washed cells
are plated on a collagen-treated substratum in DMEM +
20% plasma-derived horse serum (PDHS) + 150 μ g/ml
endothelial cell growth supplement (ECGS, available
20 from Sigma Chemical Co., St. Louis, MO); (McGuire,
P.G., et al., Lab. Invest., 57:94 (1987)).

To prepare mixed bovine aortic endothelial cells,
aortas were trimmed of adventitia and connective
tissue, cut open to expose the intimal layer, and the
25 internal aspect contacted with 0.1% collagenase in RPMI
1640. After incubation for 20 minutes at 37°C, the
loosened cells were scraped into DMEM + 10% fetal calf
serum (FCS) and plated into tissue culture flasks. For
rodent aortic endothelial cells, the exposed intima
30 were placed on a collagen-treated surface in a minimal
quantity of growth medium (DMEM + 20% FCS + 150 μ g/ml
ECGS); endothelial cells will grow out from the explant
and proliferate in this growth medium.

0 Mixed populations of human endothelial cells may
also be isolated from fresh umbilical veins. After
cannulating the vein and flushing it with RPMI 1640
medium, the intimal layer is exposed to 1 mg/ml
collagenase in RPMI 1640. After 15 minutes at about
5 37°C, the detached cells are washed out of the vein,
pelleted by centrifugation, the cell pellet suspended
in DMEM + 20% FCS, and the cells plated on a collagen-
treated substratum (Gimbrone, M.A., et al., J. Cell
Biol., 60:623 (1974)). These cells are also available
10 commercially (Clonetics, San Diego, CA).

Cells are identified as endothelial by
immunofluorescence assay with anti-von Willebrand
protein (rabbit serum from Bering Diagnostics, La
Jolla, CA) and uptake of di-I-labeled acetylated LDL
15 (Molecular Probes, Junction City, OR). Endothelial
cells are typically passaged once a week and maintained
in DMEM + 10% or 20% FCS or 10% PDHS.

Endothelial cell cultures can be cloned, if
desired, using the cloning ring technique. Cells are
20 plated in at low density (1000 cells per 10 cm plate)
in 10% FCS. Plastic cloning rings, dipped in silicone
grease, are paced on cells so as to encircle and
isolate single or paired cells on an inverted
microscope. Once the clone expands, the cells are
25 detached by trypsinization within the ring and
transferred to a well of a multi-well culture disk.
Multiple clones of microvascular endothelial cells from
bovine brain, bovine aorta, rat aorta, and rat brain
can be isolated by this technique.

30 Astrocyte-Derived Conditioned Medium

Neonatal rat brain type 1 astrocytes were grown to
confluency in poly-D-lysine-coated 75 cm² flasks.

- 0 Fresh medium was added to the cells, and removed after 2-4 days. The medium was filtered through a 0.2 μ Millipore filter, and stored frozen at -80°C in small aliquots.

Endothelial Cell-Derived Conditioned Medium

- 5 Bovine aortic or retinal endothelial cells were grown to confluency in 75 cm² flasks. Fresh medium was added to the cells, and conditioned medium collected and stored as above.

Astrocyte Extract

- 10 Type 1 astrocytes from neonatal rat brain were grown as above. Cells were scraped from the dish in 3 ml of ice-cold DMEM, and homogenized in a Dounce homogenizer at ice-bath temperatures. After centrifuging the homogenate at 40,000 rpm for 30
15 minutes in a Beckman Instruments SW40 rotor, the supernatant fluid was filtered through a 0.2 μ Millipore filter, and small aliquots stored frozen at -80°C.

Brain Extract

- 20 Neonatal rat brain cortex was removed and homogenized in DMEM (3 ml per gm. tissue, wet wt.) in a Dounce homogenizer. The homogenate was centrifuged and processed as for the astrocyte extract above.

Elevation of Cyclic AMP Concentrations

- 25 Cultures of endothelial cells were treated with one or more agents known to increase cyclic AMP concentrations. These include, but are not limited to:
1) from about 10 to about 100 μ M of a β -adrenergic agent, such as isoproterenol, that binds to specific

0 β -adrenergic receptors on cell surfaces and stimulates
G-protein-mediated activation of adenylate cyclase; 2)
serotonergic compounds such as 5-hydroxytryptamine; 3)
forskolin, (Sigma Chem. Co., St. Louis, MO) an agent
that directly activates adenylate cyclase; 4)
5 parathyroid hormone; and 5) calcitonin gene related
peptide. Adding an inhibitor of cyclic AMP
phosphodiesterase, the enzyme that degrades cyclic AMP
to adenylic acid, will accentuate the cyclic AMP
elevating effects of the aforementioned modalities;
10 examples of such inhibitors are 4-(3-butoxy-4-
methoxybenzyl)-2-imidazolidinone (Hoffman-LaRoche,
Nutley, N.J.), theophylline and methylisobutylxanthine
(Sigma Chem. Co.), Rolipram (Berlex, Inc.) and
RO-20-1724 (BioMol, Inc., Phymouth Meeting, PA). In
15 addition, certain derivatives of cyclic AMP can be used
to elevate the effective cyclic AMP concentration in
such cells; such derivatives include 8-bromo cyclic AMP
(Sigma Chem. Co) and 8-(4-chlorophenylthio)cyclic AMP
(Boehringer-Mannheim Corp., Indianapolis, IN). By
20 "effective cyclic AMP" we mean endogenous cyclic AMP or
cyclic AMP derivatives to which endothelial cells are
permeable and which act physiologically as does
endogenous cyclic AMP within such cells. By "effective
cyclic GMP" we mean endogenous cyclic GMP or cyclic GMP
25 derivatives to which endothelial cells are permeable
and which act as does endogenous cyclic GMP within such
cells. By "physiological action" of cyclic AMP or
cyclic GMP or derivatives thereof we mean those
immediate biochemical reactions of these cyclic
30 nucleotides that lead ultimately to the physiological
actions ascribed to them. For example, cyclic AMP
activates certain protein kinases that catalyze the
phosphorylation of hydroxyamino acid residues such as

0 serine, threonine and tyrosine in particular proteins,
such phosphorylation activating these proteins.
Effects of cyclic AMP are reversed by phosphoprotein
phosphatases that catalyze the de-phosphorylation of
the aforementioned hydroxyamino acid residue-containing
5 proteins.

When brain capillary endothelial cells were grown
on a porous solid support with a growth medium
containing PDHS plus one or more of the aforementioned
agents that elevated the actual or effective
10 intracellular cyclic AMP concentration, transmonolayer
electrical resistance increased about 7-fold, from
about 50 to about 350 ohm-cm² (Fig. 1). However, when
in this system, bovine aortic endothelial cell-derived
conditioned medium (BAEC-CM) was also present, the
15 transmonolayer electrical resistance increased about
10-fold (Example 5). Growing endothelial cell
monolayers on astrocyte extracellular matrix
potentiated the effects of cyclic AMP and BAEC-CM,
producing as much as a 26-fold increase in resistance
20 (Example 5). Thus, the BBB model of this invention is
capable of providing transmonolayer electrical
resistances of at least 200 ohm-cm², preferably greater
than about 300 ohm-cm², more preferably greater than
about 1000 ohm-cm² up to about 1500-2000 ohm-cm².

25 In addition, it has been discovered that an
elevation of actual or effective cyclic AMP
concentrations, with consequent formation of tight
junctions between mixed endothelial cells as determined
by electrical resistance measurements, was also
30 associated with substantial peripheral staining by
phalloidin, a toxin produced by Amanita phalloides that
is known to bind to filamentous actin and prevent their
depolymerization (Stryer, L., "Biochemistry", 3d., W.H.

0 Freeman, N.Y. 1988, p. 940). The belt-like pattern of
phalloidin staining in these treated endothelial cells
is similar to that seen in epithelial cells exhibiting
high resistance tight junctions. (Gumbiner, B., J.
5 Cell Biol., 107:1575 (1985).) In addition, when
endothelial cells were grown with endothelial cell-
derived or astrocyte-derived conditioned medium plus
cyclic AMP enhancing agents such that peripheral
phalloidin staining of cells was substantially present,
transmonolayer electrical resistance was increased over
10 that obtained in the absence of conditioned medium.

It has also been discovered that the formation of
tight junctions between endothelial cells in the blood-
brain barrier model of the invention is substantially
enhanced when endothelial cells are grown from the time
15 of their isolation in the presence of astrocyte-derived
conditioned medium (ADCM). Thus, when endothelial
cells are passaged onto filters, e.g., Costar filters,
they are preferably grown in a medium containing 50%
ADCM made in MEM with 10% fetal calf serum and 50% N2
20 (a chemically-defined medium). After 2-3 days of
growth on the filters, they may be treated with a
cyclic AMP analogue and a cyclic AMP phosphodiesterase
inhibitor (e.g., Rolipram or RO 20-1724).

In addition, it has been discovered that, when
25 endothelial cells are cultured in concentrations of
fetal calf serum substantially lower (e.g., 0.5% to 5%)
than the customary 10% used for culturing such cells
for other purposes, increased cellular resistivity in
the blood-brain model of the invention may be attained.

0 Decreased Cyclic AMP Concentrations
 or Physiological Activity

 As noted above, increases in cyclic AMP in tissues by whatever means (e.g., addition of cyclic AMP analogues, addition of compounds that stimulate
5 endogenous adenylate cyclase activity, or addition of compounds that inhibit the activity of cyclic AMP phosphodiesterase thereby inhibiting cyclic AMP degradation) result in enhanced tight junction formation between brain endothelial cells.

10 It has also been discovered that removal of cyclic AMP analogues or other agents that elevate cyclic AMP levels from endothelial cell cultures in the blood-brain model of the invention produces a rapid decrease in resistance, signalling increased permeability of
15 tight junctions.

 These discoveries have led to other approaches to regulating tight junctions between brain endothelial cells: (a) compounds that inhibit formation of endogenous cyclic AMP by adenylate cyclase; (b)
20 competitive inhibitors of cyclic AMP; (c) inhibitors of protein kinases, the enzymes that are activated by cyclic AMP; and (d) stimulators of protein phosphatases, the enzymes that dephosphorylate proteins that had been phosphorylated, and thereby activated, by
25 the cyclic AMP system.

 The basis of approach (a) above is as follows. A system for the regulation of adenylate cyclase activity in plasma membranes consists of GTP; a G_i regulator protein which, when bound to GTP, inhibits the activity
30 of adenylate cyclase; a G_s regulatory protein which, when bound to GTP, activates adenylate cyclase; and, agonists that increase the binding of GTP to G_i or G_s . It has now been discovered that agonists that increase

0 the binding of GTP to G_i , such as α -adrenergic agents
and adenosine A1 receptor agonists [e.g.,
cyclopentyladenosine (CPA) and the (-) stereoisomer of
N⁶-(phenylisopropyl)-adenosine (R-PIA)], particularly
5 the latter agonists, are effective in reducing the
resistance of brain endothelial cells. Such
observations may be made in vitro in the blood-brain
model of the invention or in vivo in brain infusion and
behavioral test systems in mice, as shown in the in
10 vivo examples below. For example, it was found that G_i
agonists that presumably inhibited the cellular
production of cyclic AMP lowered the amount of morphine
that had to be administered intravenously to mice in
order to produce analgesia (morphine does not penetrate
well into the brain). Another in vivo test system
15 comprises the intravenous administration of a test drug
to an unanesthetized animal, the injectate also
containing labeled tracer substances that normally do
not penetrate the BBB. Thereafter, the test animal is
injected with an anesthetic, followed by phosphate-
20 buffered saline and a tissue fixative. The brain is
then removed and dissected, and the amount of tracer
substance quantified. Observation with these test
systems suggest that agents that inhibit adenylate
cyclase and reduce cyclic AMP production increase the
25 permeability of tight junctions and open up the blood-
brain barrier, thereby providing a drug delivery
system.

Approach (a) above also includes the use of
inhibitors that block the binding to the receptors for
30 the aforementioned G_s system of endogenous ligands,
e.g., norepinephrine, that stimulate the G_s system. By
this means, endogenous production of cyclic AMP is
reduced, thereby reducing tight junction formation

0 between brain microvascular endothelial cells.
Approach (a) also includes agents that directly inhibit
adenylate cyclase, such as the synthetic nucleoside
dideoxyadenosine.

 The basis of approach (b) above is that
5 competitive inhibitors of the action of cyclic AMP will
increase the permeability of tight junctions, thereby
opening up the blood-brain barrier. Compounds of this
type that can be tested in the blood-brain model of the
invention include the R_p diastereoisomer of cyclic AMP.

10 The basis of approach (c) above is as follows.
Cyclic AMP is known to act physiologically by
activating one or more protein kinases that, in turn,
catalyze the phosphorylation of key proteins. Thus,
inhibitors of protein kinases should nullify the
15 effects of cyclic AMP on tight junction formation
between brain endothelial cells. As will be detailed
in the examples below, protein kinase inhibitors such
as K252a and staurosporine at nM (10-200 nM)
concentrations can markedly reduce the resistance of
20 brain endothelial cell cultures. Both inhibitors were
reversible. At the light microscope level, it was
discovered that either removing cyclic AMP or adding a
protein kinase inhibitor caused clear separations of
endothelial cell tight junctions.

25 The basis of approach (d) above is that
dephosphorylation of those key proteins whose
phosphorylation had been catalyzed by cyclic AMP-
activated protein kinases will produce an
unphosphorylated protein that is inactive in
30 maintaining tight junctions among endothelial cells.

0 Increased Cyclic GMP Concentrations or Activity

Cyclic GMP, another regulatory cyclic nucleotide, is produced from GTP by the enzyme guanylate cyclase.

It has now been discovered that increasing cyclic GMP concentrations or physiological activity in brain
5 endothelial cells leads to a decrease in resistance, and thus to an increase in tight junction permeability. Increased concentrations or physiological activity of brain endothelial cell cyclic GMP can be achieved by,
10 for example, 8-bromo-cyclic GMP, atrial natriuretic factor and sodium nitroprusside, and by cyclic GMP phosphodiesterase inhibitors such as dipyridamole (Research Biochemicals, Inc.) or Zaprinast (Rhone-Poulenc). It was observed, for example, that
15 nitroprusside at concentrations of from 0.1 to 100 μ M markedly inhibited the effect of a cyclic AMP analogue, RO-20-1724, on elevating the resistance of brain endothelial cells in the blood-brain model of the invention. Such agents may be used in the
20 aforementioned in vivo test systems to determine their effect on the opening up the blood brain barrier in test animals. For example, the in vivo morphine analgesia test system described below can be used to demonstrate that sodium nitroprusside opened up the blood-brain barrier to morphine, and that dipyridamole
25 opened up the blood-brain barrier to enkephalin, an endogenous opiate in vertebrates that does not penetrate significantly into the brain when administered in the peripheral circulation.

30 Construction of a Chamber BBB Model

In a general embodiment of this invention, brain capillary endothelial cells are grown on a porous substratum-coated solid support, e.g., filters or

0 membranes. It has been found that endothelial cells
can attach to and grow on Nucleopore polycarbonate
filters (Costar, Inc., Cambridge, MA), Millicell CM and
HA porous nitrocellulose filters (Millipore Corp,
Bedford, MA), and collagen membranes (ICN Biomedical,
5 Inc., Costa Mesa, CA). The Millicell CM and Nucleopore
polycarbonate filters required pre-treatment, i.e.,
coating, with extracellular matrix material (ECM, see
below), components in order to promote adhesion of
cells to the filter. Nucleopore filters promote media
10 exchange across the filter, and permit cellular
processes to cross through. Filters allow cells more
completely to establish blood side and brain side
domains, as they permit separate manipulation of the
two compartments of the chamber.

15 Porous solid supports can be coated with ECM by
soaking them in an aqueous solution of laminin,
vitronectin, or fibronectin (typically, from about 10
to about 50 $\mu\text{g/ml}$), Matrigel^R (an extract of EHS
sarcoma obtainable from Collaborative Res., Bedford,
20 MA) in PBS, type I rat tail collagen or type IV
collagen in dilute acetic acid (Collaborative Research,
Inc., Collagen Corp, and New York Blood Bank, N.Y.), or
astrocyte extracellular matrix (AECM).

In a preferred embodiment, filters were coated
25 with astrocyte extracellular matrix (AECM) synthesized
by astrocytes, in the following manner. Rat brain type
I astrocytes, produced as described above, were grown
on filters in a chemically-defined medium. Once cells
reached confluence, they were lysed by a low ionic
30 strength buffer containing a nonionic detergent, e.g.,
Triton X-100, and rinsed with PBS containing a protease
inhibitor such as aprotinin. This removed the cells
and left behind AECM as a coating on the filter.

0 Alternatively, AECM was generated by solubilizing the
cells of a confluent monolayer of astrocytes with
nonionic detergent, and then extracting residual
extracellular matrix components with a denaturant such
as 6 M urea, 6 M guanidine HCl or 2 M MgCl_2 . This
5 extract was dialyzed against physiological saline
before adding to endothelial cells being cultured on
filters or used as a filter coat prior to adding
endothelial cells.

In one embodiment of the chamber-type in vitro
10 model of the BBB of this invention, an essentially
confluent monolayer of rat brain type 1 astrocytes was
disposed on one side of a porous solid support, and an
essentially confluent monolayer of endothelial cells
was disposed atop an ECM coating on the opposite side
15 of the porous solid support. The thus-obtained
co-culture device was then affixed in a chamber,
effectively dividing the chamber into at least two
compartments, one of which represents the brain side
(endothelial cell side) and the other the blood side of
20 a BBB. The cells were then placed in contact with a
growth medium, preferably containing PDHS.

In another embodiment, an ECM-coated porous solid
support was affixed in a chamber. On one ECM-coated
surface of the porous solid support there was disposed
25 a culture of astrocytes; endothelial cells were then
plated at low density (about 10^5 cells/30 mm filter)
onto the opposite ECM-coated surface. The astrocytes
"condition" the growth medium that contacts the
endothelial cells and may induce appropriate
30 differentiative changes in the endothelial cells,
including formation of tight junctions. The cells were
grown in a culture medium containing serum, preferably
PDHS.

0 In another embodiment, microvascular endothelial
cells were disposed on an uncoated or EMC-coated porous
solid support as described above, and the support was
affixed in a chamber, on a surface of which there was
disposed a culture of brain astrocytes. The growth
5 medium must contact both the endothelial cells and
astrocytes to effect biochemical interaction between
the co-cultures.

 In yet another embodiment, microvascular
endothelial cells were disposed on an ECM-coated porous
10 solid support as described above, but astrocytes were
absent from either the contralateral side of the porous
solid support or from a surface of the chamber.
Instead, the growth medium in the blood compartment of
the chamber, i.e., the compartment opposite that which
15 houses endothelial cells, was supplemented with from 0%
to 100% with astrocyte-derived or endothelial cell-
derived conditioned media, or with brain or other
tissue extracts, obtained as described above, as
required.

20 Agents that are intended to elevate intracellular
concentrations of cyclic AMP in endothelial cells or to
increase the concentration of effective cyclic AMP may
be added to the growth medium, as can be dyes, e.g.,
trypan blue or Evans blue, or other macromolecules that
25 are used to test for tight junction resistance.

 It is preferred that the glucose concentration of
a growth medium in contact with endothelial cells not
be higher than physiological in vertebrates, i.e.,
approximately 100 mg/dl.

30 Assay for Tight Junctions

 The presence of tight junctions in the endothelial
layer of the BBB model can be detected using reagents

0 that recognize proteins associated with tight
junctions. For example, the monoclonal antibody 40.76,
made against ZO-1 tight junction protein, specifically
recognizes an antigen on both bovine and mouse
endothelial cells (Anderson, J.M. et al., J. Cell
5 Biol., 106:1141 (1988); Stevenson, R.B., et al., J.
Cell Biol., 103:755 (1986)). This approach allows the
user to detect the formation of tight junctions among
small subsets of endothelial cells, and to refine
culture conditions to enhance the formation of tight
10 junctions.

The degree of tightness of tight junctions can be
also assessed by transcellular electrical resistance
measurements. For transendothelial cell resistance
measurements, cells were grown on a porous solid
15 support, e.g., a filter or membrane attached to a
holding device in order adequately to suspend the
cellular monolayer, such as the Costar Transwell
apparatus or the ICN Cellogren. Transmonolayer
resistance is measured, for example, with the device of
20 Perkins et al. (Perkins, F.M., et al., Am. J. Physiol.,
241:C154 (1981)). Cells were maintained in a growth
medium or physiological saline, and calomel electrodes
on each of the endothelial cells are connected by a
saturated KCl - 3% agar bridge. Current is passed
25 between two Ag-AgCl electrodes and the voltage measured
with a Keithly multimeter. Resistance is calculated
from the change in voltage across the monolayer induced
when a short current pulse (10-100 μ amp) is applied.
The resistance of the filter or membrane alone is
30 subtracted. The resistance, multiplied by the surface
area of the filter or membrane, yields the resistance
in ohms-cm².

0 As noted above, peripheral binding of the toxin
phalloidin reveals the presence of belt-like
filamentous actin, a hallmark of tight junction
formation among endothelial cells. Staining of
filamentous actin by phalloidin can be visualized using
5 derivatives such as phalloidin coumarin
phenylisothiocyanate or fluorescent FITC-phalloidin or
TRITC-phalloidin (Sigma Chem. Co., St. Louis, MO).

10 Another means for assessing the formation of tight
junctions among endothelial cells is to determine the
transport of macromolecules from the apical blood side
to the abluminal brain side. For example, the water-
soluble dye Evans blue (mol. wt. 960) that binds
strongly to albumin (Freedman, F.B., et al., Am. J.
Physiol., 216:675 (1969)), can be used to assess the
15 tightness of newly formed endothelial cell junctions;
tissues with tight junctions that exclude the dye or
exhibit limited transport will remain white, whereas
those without tight junctions or that exhibit
significant transport capabilities will be stained blue
20 as the dye passes through the junctions. Other water-
soluble, macromolecular markers for tight junction
formation include fluorescein isothiocyanate bound to
dextran (FITC-dextran. mol. wt. 20,000, Sigma Chem.
Co.) and ¹²⁵I-labeled albumin (DuPont/NEN, Wilmington,
25 DE). Fluorescent dextrans of other sizes and sodium
fluorescein itself may be used as well.

Still another means for assessing the tightness of
junctions among endothelial cells in the blood-brain
model of the invention is to compare the transport of a
30 hydrophilic compound, e.g., sucrose, and a hydrophobic
compound of similar size, e.g., chlorambucil, across
filters with and without monolayered endothelial cells.
When the transmonolayer resistance is high, the

- 0 transport of sucrose should be low compared to that of
chlorambucil (or other hydrophobic compounds of similar
size). Alternatively, when the resistance is high, the
transport of sucrose should be much less (e.g., 50-fold
or more) than across cell-free filters. Contrariwise,
5 in "leaky" cell junctions, the relative transport of
sucrose will be substantially increased.

Assessment of Ligand Binding, Transcytosis
and Drug Delivery

- The access in the model of the invention to both
10 sides of a differentiated endothelium or ECM-coated
porous solid supports permits the assay of specific
binding and uptake of radiolabeled ligands from an
apical (luminal) or basolateral (abluminal) aspect.
Furthermore, by adding a labeled probe to one side of
15 the porous solid support, one can assess the ability of
the probe to be transcytosed from one side of the
monolayer to the other.

- The model also allows for testing the access of
potential new therapeutics to the brain parenchyma.
20 For example, drugs such as L-DOPA can cross the BBB,
being recognized and transported by amino acid
transporters. Lipophilic drugs are also able to
penetrate the BBB. However, as indicated above,
potentially therapeutic drugs that are not lipophilic
25 and for which no specific transport mechanism exists
may be unable to penetrate the BBB or may do so at
rates insufficient to maintain a therapeutic drug level
in the brain. The in vitro model of the BBB of this
invention can also be used to test tight junction-
30 disrupting compositions. It has been found by
immunohistological methods that a molecule
immunologically related to the mouse cell-adhesion

- 0 molecule E-cadherin is present on mouse endothelial cells. The expression of the E-cadherin-like molecule is enhanced in cultures of brain endothelial cells exhibiting increased resistance (Example 9).

Vasogenic Brain Edema

- 5 It is generally thought that brain edema is caused by increased tight junction permeability (decreased resistance) and/or increased pinocytosis. To the extent to which enhanced tight junction is important, agents that increase cyclic AMP concentration or
10 physiological activity in brain microvascular endothelial cells may be of therapeutic value. These include cyclic AMP analogues, agonists that bind to receptors coupled to the Gs regulatory protein, adenylate cyclase activators, cyclic AMP-specific
15 phosphodiesterase inhibitors, protein phosphatase inhibitors, and protein kinase stimulators. We have discovered, in this connection, that the phosphodiesterase primarily responsible for degrading cyclic AMP in brain microvascular endothelial cells is
20 a member of the class termed the Type III cyclic GMP-noninhibitable phosphodiesterase, and that this enzyme is inhibited by compounds such as Rolipram and RO-20-1724 mentioned above.

Other Uses of the Model

- 25 While the foregoing illustrates preferred modes of practicing this invention, other embodiments of the basic concepts of the present invention can also be practiced. For example, the model can be used with cocultures of pulmonary or aortic arterial endothelial
30 cells, with other cells of vascular walls, e.g., smooth muscle cells, in order to study morphological and

0 metabolic interactions between the two types of cells,
as well as transcellular transport and drug
permeability. The model may also be used as a
chemotaxis chamber to study brain migration of
5 lymphocytic cells through monolayers of brain
endothelial cells to analyze CNS diseases such as
multiple sclerosis. Yet another use of the model is to
test other endothelial cell barriers, such as in the
testes and retina. Still another use of this model is
10 to screen reagents useful to prevent or ameliorate
brain inflammation, such as in the embodiment described
below wherein the model is used to screen compositions
for the modulation of the adhesion of white blood cells
to brain endothelia.

MODULATION OF LEUKOCYTE ADHESION
TO BRAIN ENDOTHELIAL CELLS

15

The present blood brain barrier model was also
used to screen reagents useful to prevent or ameliorate
brain inflammation. The blood brain barrier model was
used to select reagents that prevent the attachment of
20 inflammatory white blood cells to the brain
endothelium. This model, in conjunction with analysis
of brain tissue, allowed identification of one of the
receptors that white blood cells use to adhere to brain
endothelial cells. Once this receptor was identified,
25 reagents and methods useful for ameliorating or
preventing inflammation were ascertained, as were
therapeutic compositions useful for treating brain-
inflammatory disease such as multiple sclerosis.

White blood cells (leukocytes) travel continuously
30 in the general circulation. At the site of an injury
or other inflammatory stimulus, cells that line blood
vessels (endothelial cells) become activated to express

0 molecules that are adhesive for leukocytes. Thus,
following an inflammatory stimulus, leukocytes bind to
the activated endothelium. Once bound, the leukocytes
migrate across the blood vessel wall to enter the
injured site and release toxic mediators to combat
5 infection. Unfortunately, the leukocyte toxins can also
cause indiscriminate tissue damage. Such is the case
with multiple sclerosis (MS). In MS, large numbers of
leukocytes leave the blood stream within the brain and
cause extensive tissue damage. See Hickey, W.F.,
10 Psychoneuroimmunology II, Academic Press (1990).

In order for leukocytes to enter any tissue, they
must first bind to the vascular endothelium. It has
been shown in other disease systems that, in spite of
the initial insult, if leukocyte binding to the
15 endothelium at the damaged site is inhibited, then the
leukocytes do not enter the tissue and further damage
is greatly avoided. Simpson et al., J.Clin.Invest. 81:
624-629 (1988) disclose that the administration of a
monoclonal antibody that binds to a leukocyte cell
20 adhesion-promoting glycoprotein (Mol; CD11b/CD18)
resulted in reduced injury to heart tissue because
fewer leukocytes (neutrophils) bound to the heart
tissue.

The mechanics of leukocyte adhesion to endothelial
25 cells involves, in part, the binding of cell surface
receptors on leukocytes to the corresponding cell
surface receptors on endothelia. Both leukocytes and
endothelial cells are known to express various
adhesion-promoting receptors at various times in
30 response to various stimuli. For reviews of adhesion
receptors of the immune system, see generally,
Springer, Nature 346: 425 -434 (1990), and Osborn, Cell
62: 3-6 (1990) both of which are herein incorporated by

0 reference. The expression of cell adhesion molecules is
not predictable, and may vary widely in response to
different inflammatory stimuli and in different
anatomical locations. For example, Tuomanen et al., J.
5 Exp. Med. 170: 959-968 (1989) show that antibodies
directed against the CD18 family of adhesion-promoting
receptors blocks the migration of leukocytes across the
blood brain barrier in response to acute inflammatory
stimulus of bacterial origin. Anti-CD18 was shown to
not block leukocyte migration to the lung. Vedder et
10 al., Surgery 106: 509 (1989).

Circulating leukocytes may express the VLA-4
receptor, and this has been show to bind to the VCAM-1
receptor on cytokine-activated human endothelial cells.
Elices, et al., Cell 60: 577-584 (1990). The different
15 types of molecules induced on blood brain barrier
endothelial cells during brain inflammation, and the
role that they play in chronic inflammatory brain
disease, such as MS, is poorly understood.

A. Ameliorating or Preventing Brain Inflammation

20 The present embodiment was directed toward finding
reagents that modulate leukocyte adhesion in the brain.
The blood brain barrier model of the present invention
was one system used. Using this model, brain
endothelial cell samples, prepared in accordance with
25 the present description, were activated with
inflammation mediators. To a panel of these activated
cell samples, leukocytes were introduced in the
presence of a different putative receptor-blocker for
each sample. Individual samples were assayed for the
30 presence or degree of leukocyte adhesion. Here, among
the various reagents tested, two antibodies directed
against VLA-4 (the leukocyte cell adhesion molecule)

0 were shown to block lymphocyte binding to brain endothelium.

A separate assay produced the same results. Essentially, slices of brain tissue were analyzed for their ability to bind leukocytes in the presence of
5 putative cell adhesion modulators. In this system, another novel aspect of the present invention was developed. Rats were injected with human tumor cells in order to induce inflammation in the brain. Previously, it was not known that this method could induce traffic
10 across the blood brain barrier into the brain. Further, the type of inflammation induced is very much like that seen in multiple sclerosis victims where inflammation is characterized by small vessels, with activated almost cuboidal endothelium. The vessels appear very
15 similar to the "high endothelial venules" seen in lymphoid tissues. Further, the vessels are surrounded by a cusp of lymphocytes, and active lymphocyte traffic is apparent. While MS-type inflammation has been observed, it has never before been induced by this
20 method. Thus, induction of brain inflammation using tumor cells possesses great utility in obtaining tissue for an in vitro model of multiple sclerosis.

After an appropriate length of time, rat brains in which inflammation had been induced were removed and
25 sectioned. To these sections, leukocytes were added, in the presence of the putative cell adhesion modulator to be screened. Here too it was found that the anti-VLA-4 antibodies inhibited leukocyte adhesion.

This inhibition is graphically illustrated in the
30 Figures. Figure 8, Panel A shows a brain section in which no antibody is added. The small dark dots are leukocytes against the background of inflamed brain endothelial cells. As can be seen, the leukocytes are

0 quite densely bound to the vessels in inflamed tissue.
Figure 8, Panel B shows inhibition of binding by
antibodies directed against the $\beta 1$ subunit of VLA-4.
Figure 9 shows a brain endothelial culture to which
lymphocytes have bound. Panel A illustrates binding to
5 unstimulated endothelium. Panel B shows binding to
endothelium stimulated for twelve hours by $\text{TNF}\alpha$. In
Panel C, the lymphocytes have been pretreated with
anti- β -1 integrin and their binding to stimulated
endothelium is greatly inhibited. As described in
10 Example 21, below, the binding density of human
leukocytes to brain sections was confirmed by using an
internal population of mouse leukocytes, a population
not recognized by the anti-human reagent. This
quantification confirmed the visual observation that
15 anti-VLA antibodies prevented leukocyte binding to
brain sections in which multiple sclerotic-type
inflammation had been induced. (Figure 10). Further,
leukocyte binding to cultured endothelium was
quantified by prelabeling the cells with ^{125}I ; the
20 inhibitory effects of anti- β are illustrated in
Figure 11.

Different cell adhesion molecules are expressed in
different tissues in response to a variety of stimuli.
Brain-specificity may be beneficial in administering a
25 leukocyte adhesion modulator for therapeutic purposes.
The VLA-4 leukocyte adhesion molecule is known to be
expressed throughout the body under various conditions.
Other tissues, apart from brain tissue, were analyzed
in order to determine if either the anti- α -4 or the
30 anti- β -1 antibody had any immune reaction in those
tissues. As shown in more detail in Example 22, the
anti- α -4 inhibited lymphocyte binding to normal
intestinal lymphoid tissue, but did not affect binding

0 to normal lymph nodes. The anti- β -1 antibody does not inhibit binding to lymph nodes and would not be expected to affect binding to intestinal lymphoid tissue.

5 VLA-4 is a member of the β 1 integrin family of cell adhesion molecules, each of which is comprised of two subunits, an α and a β chain. There are at least six β 1 integrins, all sharing the same β 1 chain and each having a distinct α chain. These six receptors all bind a different complement of the various cell
10 matrix molecules, such as fibronectin, laminin, and collagen. VLA-4, for example, binds to fibronectin. VLA-4, however, is unique in that it also binds to a non-matrix molecule that is expressed by endothelial cells. This molecule is called VCAM-1, and is thought
15 to be expressed on endothelium at various geographic locations in response to various stimuli. Distinct epitopes of VLA-4 are responsible for the fibronectin and VCAM-1 binding activities, and each activity can be inhibited independently.

20 One of the monoclonal antibodies presently used, HP2/1 reacts with the α chain of VLA-4 and blocks only its binding to VCAM-1. It does not affect binding of VLA-4 to fibronectin, nor does it affect the activity of the other members of the β 1 integrin family.

25 However, the α chain of VLA-4 also interacts with a distinct β chain, called β p. This receptor mediates all lymphocyte binding to intestinal lymphoid tissues.

Another of the antibodies used, the monoclonal antibody, HP2/1, reacting with VLA-4 α blocks the
30 activity of this molecule, i.e., it prevents the binding of VLA4 $\alpha\beta$ p to intestinal endothelium (illustrated in Table 12). The monoclonal antibody, AIIB2 reacts with the β 1 chain that is common to all

0 members of the $\beta 1$ integrins and potentially
immunoreacts with the entire family, including the
fibronectin and the VCAM-1 binding activities of VLA-4.
It would not be expected to inhibit lymphocyte binding
to intestinal endothelium, however, because it would
5 not bind to βp .

Reagents which selectively react against the VLA-
4/VCAM-1 target are also envisioned. For example, an
antibody which interacts with the VCAM-1 binding domain
of VLA4 α in conjunction with the $\beta 1$ chain would block
10 only lymphocyte migration into sites of inflammation,
such as the brain during multiple sclerosis. This
reagent further would not affect matrix interactions
(mediated by all members of the $\beta 1$ integrins) nor would
it affect normal intestinal immunity (mediated by VLA-
15 4 $\alpha\beta p$). The production of this, and other such reagents
is well within the skill of the art.

B. VLA-4/VCAM-1 Directed Cell
Adhesion Modulators And Uses

The VLA-4/VCAM-1 molecules, instrumental in brain
20 inflammation (particularly MS-type brain inflammation)
provide molecular targets that can be put to a variety
of uses. The present invention thus encompasses these
uses and related compositions.

First, as is shown by Example 23, receptors
25 against the VLA-4 ligand may be used to modulate
leukocyte adhesion to brain endothelial cells. Herein,
the term "receptor" is used to denote a biologically
active molecule that binds to a ligand. For example,
antibodies or fragments thereof, which immunoreact with
30 the VLA-4 molecule may be useful to prevent leukocyte
binding to brain endothelial cells. Peptides, or
peptidomimetics or related compounds, which can act to
bind the cellular adhesion molecule, are also

0 contemplated, and these may be made synthetically by
methods known in the art. Other receptors which react
with a VLA-4 ligand will be apparent to those skilled
in the art.

5 Additionally, receptors against a VCAM-1 ligand
may be used to modulate leukocyte adhesion to brain
endothelial cells. Either way, one cell adhesion
molecule is blocked, and one pathway of leukocyte
adhesion is terminated.

10 It should be recognized that for therapeutic
purposes, therapeutically effective compositions for
preventing or ameliorating brain inflammation
containing such VLA-4 or VCAM-1 directed receptors are
contemplated as within the scope of the present
invention. For example, therapeutic compositions
15 including at least one VLA-4 receptor or VCAM-1
receptor as well as other therapeutic compositions
could be used to prevent or ameliorate inflammation of
brain endothelial cells. Another example is the use of
a VCAM-1 receptor, to which is attached a drug useful
20 for treating MS or other inflammatory condition, for a
drug delivery vehicle which also prevents the adhesion
of leukocytes to the VCAM-1 molecule. Peptides or
peptidomimetics or other molecules, which serve to
substantially mimic one cell adhesion molecule or the
25 other, could be used in competition therapy wherein
such peptides or peptidomimetics (or other compounds)
compete for the available locations on the surface of
either the leukocyte (if substantially mimicking VCAM-
1) or the endothelial cell (if substantially mimicking
30 VLA-4).

Suitable pharmaceutical carriers and their
formulations are described in Martin, Remington's
Pharmaceutical Sciences, 15th Ed. (Mack Publishing Co.,

0 Easton 1975). Such compositions will, in general,
contain an effective amount of the active compound
together with a suitable amount of carrier so as to
prepare the proper dosage form for proper
administration to the host. Useful pharmaceutical
5 carriers for the preparation of the pharmaceutical
compositions hereof can be solids, liquids or gases;
thus, the compositions can take the form of tablets,
pills, capsules, powders, enterically coated or other
protected formulations (such as by binding on ion
10 exchange resins or other carriers, or packaging in
lipid protein vesicles or adding additional terminal
amino acids), sustained release formulations, solutions
(e.g., ophthalmic drops), suspensions, elixirs,
aerosols, and the like. Water, saline, aqueous
15 dextrose, and glycols are preferred liquid carriers,
particularly (when isotonic) for injectable solutions.
The carrier can be selected from various oils including
those of petroleum, animal, vegetable or synthetic
origin, for example, peanut oil, soybean oil, mineral
20 oil, sesame oil, and the like. Suitable pharmaceutical
excipients include starch, cellulose, talc, glucose,
lactose, sucrose, gelatin, malt, rice, flour, chalk,
silica gel, magnesium stearate, sodium stearate,
glycerol monostearate, sodium chloride, dried skim
25 milk, glycerol, propylene glycol, water, ethanol, and
the like. The compositions may be subjected to conven-
tional pharmaceutical expedients such as sterilization
and may contain conventional pharmaceutical additives
such as preservatives, stabilizing agents, wetting or
30 emulsifying agents, salts for adjusting osmotic
pressure, buffers, and the like.

In the practice of the therapeutic methods of the
present invention, an effective amount of the active

0 compound, including derivatives or salts thereof, or a
pharmaceutical composition containing the same, as
described above, is administered via any of the usual
and acceptable methods known in the art, either singly
or in combination with another compound or compounds of
5 the present invention or other pharmaceutical agents
such as anti-inflammatory agents, or other therapeutics
known to have an effect on inflammation or the like.
These compounds or compositions can thus be adminis-
tered orally, sublingually, topically (e.g., on the
10 skin or in the eyes), parenterally (e.g.,
intramuscularly, intravenously, subcutaneously or
intradermally), or by inhalation, and in the form of
either solid, liquid or gaseous dosage including
tablets, suspensions, and aerosols, as is discussed in
15 more detail above. The administration can be conducted
in single unit dosage form with continuous therapy or
in single dose therapy ad libitum.

In one preferred embodiment, the therapeutic
methods of the present invention are practiced when the
20 relief of symptoms is specifically required or perhaps
imminent; in another preferred embodiment, the method
hereof is effectively practiced as continuous or
prophylactic treatment.

In the practice of the therapeutic methods of the
25 invention, the particular dosage of pharmaceutical
composition to be administered to the subject will
depend on a variety of considerations including the
nature of the disease, the severity thereof, the
schedule of administration, the age and physical
30 characteristics of the subject, and so forth. Proper
dosages may be established using clinical approaches
familiar to the medicinal arts. It is presently
believed that dosages in the range of 0.1 to 100 mg of

0 compound per kilogram of subject body weight will be
useful, and a range of 1 to 100 mg per kg generally
preferred, where administration is by injection or
ingestion. Topical dosages may utilize formulations
containing generally as low as 0.1 mg of compound per
5 ml of liquid carrier or excipient, with multiple daily
applications being appropriate.

Imaging reagents are also contemplated. A tracer
molecule, detectable in radiographic or other imaging
techniques) could be linked to an anti-VCAM or anti-
10 VLA-4 reagent to identify areas of active leukocyte
traffic in the brain. This is useful in diagnostic
protocols and in determining the progression of the
disease or the effectiveness of therapy, for example.

Other uses, formulations, compositions, and
15 processes will be readily apparent to those skilled in
the art.

The following examples are illustrative of several
embodiments of this invention, and should not be
construed as in any way limiting the invention as
20 recited in the claims.

EXAMPLE 1

ELECTRICAL RESISTANCE OF ENDOTHELIAL CELL CULTURES TREATED WITH CYCLIC AMP

Bovine brain capillary endothelial cells were
25 grown on polycarbonate filters in a conditioned growth
medium containing either 5% or 10% PDHS. In controls,
the growth medium contained 5% PDHS (\square) or 10% PDHS
(\blacksquare). In experimental cultures, the growth medium was
supplemented with either 5% PDHS + 250 μ M 8-(4-
30 chlorophenylthio) cyclic AMP (\bigcirc) or 10% PDHS + 250 μ M
8-(4-chlorophenylthio)cyclic AMP + 35 μ M RO-20-1724, a
cyclic AMP phosphodiesterase inhibitor (\bullet).

0 Transmonolayer electrical resistances were then
determined; these are shown in Fig. 1.

The cyclic AMP analogue alone greatly increased
transmonolayer electrical resistance, which is
indicative of tight junction formation. Resistances of
5 about 400 ohm-cm², were obtained with monolayers
treated with both the cyclic AMP analogue and an agent
(RO-20-1724) that inhibited degradation of cyclic AMP.

EXAMPLE 2

TRANSPORT OF LABELED ALBUMIN ACROSS ENDOTHELIAL 10 CELLS AS A FUNCTION OF TRANSMONOLAYER ELECTRICAL RESISTANCE

The flux of ¹²⁵I-labeled albumin across monolayers
of primary cultures of bovine retina endothelial cells
and of Madin-Darby Canine Kidney epithelial cells
15 disposed on 0.4 μ pore-sized polycarbonate filters was
determined as a function of tight junctions as
reflected in trans-monolayer electrical resistance of
each cell type. The results of such experiments are
plotted in Fig. 2. The height of the bars in the
20 histogram is a reflection of leakage of albumin through
the junctions between cells - the higher the bar, the
greater the leakage of albumin.

Control cell-free filter, of course, exhibited the
smallest impediment to albumin flux.

25 Substantial flux of albumin across monolayers of
bovine retina cells occurred when the electrical
resistance was only 20 ohm-cm², and this flux was
reduced by 75% in cultures exhibiting an electrical
resistance of 60 ohm-cm².

30 In contrast, the flux of albumin was virtually
abolished in monolayers of MDCK cells in which

transmonolayer electrical resistances of greater than 200 ohm-cm² were observed.

EXAMPLE 3

EFFECTS OF VARIOUS AGENTS ON

TRANSENDOTHELIAL CELL ELECTRICAL RESISTANCE

Bovine brain capillary endothelial cells were grown to substantial confluency on either 3 μ m polycarbonate or 0.4 μ m nitrocellulose filters.

Cultures were either untreated for 24 hours (control) or grown for 24 hours in a growth medium containing either 250 μ M 8-(4-chlorophenylthio) cyclic AMP + 35 μ M RO-20-1724 (cAMP) or these two agents plus 50% (w/v) bovine aortic endothelial cell-derived conditioned medium (cAMP - BAEC-CM). Transmonolayer electrical resistances were then determined. The results are shown in Table 1.

Table 1

<u>Treatment</u>	<u>Filter</u>	<u>Resistance (ohm-cm²)</u>
Control	3 μ m polycarbonate	38
cAMP	3 μ m polycarbonate	205
cAMP + BAEC-CM	3 μ m polycarbonate	348
Control	0.4 μ m nitrocellulose	94
cAMP + BAEC-CM	0.4 μ m nitrocellulose	320

The results indicate that cAMP alone produced about a 6-fold increase in transmonolayer resistance, to about 200 ohm-cm². The combination of BAEC-CM and cAMP increased resistance by about 10-fold, to about 350 ohm-cm², on polycarbonate filters.

0

EXAMPLE 4

EFFECTS OF ELEVATION OF
INTRACELLULAR CYCLIC AMP CONCENTRATION ON
TRANSENDOTHELIAL CELL ELECTRICAL RESISTANCE

5 Bovine brain capillary endothelial cells were grown essentially to confluency on 0.4 μm polycarbonate filters.

Cultures were either untreated for 24 hours (Control) or treated for 24 hours with 250 μM 8-(4-chlorophenylthio) cyclic AMP (cAMP), 10 μM isoproterenol (ISO), 10 μM 5-hydroxytryptamine (5-HT),
10 or 10 μM forskolin. In all cultures, 35 μM RO-20-1724 was also present. Transmonolayer electrical resistances were then determined. Each value in Table 2 represents the average of 3 replicate experiments,
15 referred to control cells to which was assigned a resistance value of 100.

Table 2

	<u>Treatment</u>	<u>Relative Resistance</u>
20	Control	100
	cAMP	465
	ISO	439
	5-HT	586
	Forskolin	834

25 The results indicated that any agent that elevated effective intracellular cyclic AMP concentrations enhanced transmonolayer electrical resistance by at least 4-fold. The greatest enhancement of resistance (over 8-fold) was obtained by the use of forskolin, a
30 compound that activates adenylate cyclase directly.

0

EXAMPLE 5

EFFECTS OF CYCLIC AMP, CONDITIONED MEDIUM
AND ASTROCYTE EXTRACELLULAR MATRIX ON
TRANSENDOTHELIAL CELL ELECTRICAL RESISTANCE

Bovine brain capillary endothelial cells were
5 grown to essential confluency on nitrocellulose or
polycarbonate filters. In A and B, the filters were
first coated with type I collagen and fibronectin. In
C and D, type I astrocytes from neonatal rat brain were
then grown to confluency on these collagen and
10 fibronectin-coated filters. In case C, the astrocytes
were lysed in 1% Triton X-100 in 5 mM Tris buffer, pH
7.5, for 30 min. at 25°C. The filters were rinsed
again in PBS. In case D, astrocytes were grown as in
case C, but then treated with 5 mM EDTA in PBS for 30
15 min. at 37°C to remove the cells. These filters were
also treated with 25 mM NH_4OH and rinsed in PBS.
Bovine brain endothelial cells were grown to confluency
on these different types of filters. In B, C and D,
cells were treated as in Table 1 with 250 μM 8-(4-
20 chlorophenylthio) cyclic AMP (cAMP), 35 μM 35 μM
RO-20-1724 and bovine endothelial cell conditioned
medium (BAEC-CM). Transendothelial electrical
resistances were determined; resistances were
normalized to a control value of 100 in Table 3.
25 The combination of the cyclic AMP derivative,
phosphodiesterase inhibitor and BAEC-CM produced a
substantial increase in transmonolayer electrical
resistance. These effects were further potentiated
when the cells were grown on astrocyte extracellular
30 matrix prepared as described in C. When cells were
grown on astrocyte extracellular matrix prepared as in
D, they did not grow to confluency (and, hence, the
transmonolayer resistance was low). When cells were

- 0 grown on matrices prepared from other cell types (such as endothelial cells), there was no increase in resistance.

Table 3

	<u>Treatment</u>	<u>Relative Resistance</u>
5	A) Control	100
	B) CAMP + BAEC-CM	980
	C) CAMP + BAEC-CM + astro-ECM #1	2652
	D) CAMP + BAEC-CM + astro-ECM #2	138

EXAMPLE 6

10 EFFECT OF ASTROCYTE-DERIVED CONDITIONED MEDIUM
ON ELECTRICAL RESISTANCE OF BRAIN ENDOTHELIAL CELLS

Freshly-dissociated bovine brain endothelial cells (BBEC) were plated on tissue culture dishes either in the absence or presence of astrocyte-derived
15 conditioned medium (ADCM). Cells were then plated onto collagen-fibronectin-coated filters, again in the absence or presence of ADCM. After the cells reached confluency, samples were treated with 250 μ M 8-(4-chlorophenylthio) cyclic AMP and 35 μ M RO-20-1724
20 (+cAMP in Table 4).

The data of Table 4 demonstrate that the highest resistances were obtained when cells were placed initially in ADCM.

0

Table 4

	<u>Growth Medium*</u>	<u>Plating Medium</u>	<u>Electrical resistance</u>	
			<u>- cAMP</u>	<u>+ cAMP</u>
			ohm-cm ²	
	MEM/FCS	MEM/FCS	11	42
5	50% MEM/FCS: 50% ADCM	MEM/FCS	12	142
	MEM/FCS	50% MEM/FCS: 50% ADCM	27	160
10	50% MEM/FCS: 50% ADCM	50% MEM/FCS: 50% ADCM	46	312

*MEM, minimal essential medium; FCS, fetal calf serum; ADCM, astrocyte-derived conditioned medium.

EXAMPLE 7

15

EFFECT OF FETAL CALF SERUM REDUCTION ON
ELECTRICAL RESISTANCE OF BRAIN ENDOTHELIAL CELLS

Bovine brain endothelial cells were dissociated and plated on tissue culture dishes in 50% MEM/FCS - 50% ADCM. Cells were then passaged onto collagen-fibronectin-coated filters and grown in the media indicated in Table 5. Conditions were as in Example 6, except that some cells were grown in 50% serum-free defined medium (N2). The results are shown in Table 5.

The data demonstrate that the highest resistance were attained with cells grown in ADCM and cyclic AMP analogues in the presence of reduced fetal calf serum. The same result was obtained when the defined medium N2 was replaced by MEM.

0

Table 5

<u>Treatment of cells*</u>		<u>Resistance</u> ohm-cm ²
5	MEM/FCS	40 ± 6
	+ CAMP	163 ± 22
	+ ADCM	56 ± 15
	+ CAMP + ADCM	345 ± 64
10	50% MEM/FCS, 50% N2	61 ± 2
	+ CAMP	305 ± 50
	+ ADCM	115 ± 11
	+ CAMP + ADCM	625 ± 82

* MEM, minimal essential medium; CAMP, cyclic AMP; ADCM, astrocyte-derived conditioned medium.

EXAMPLE 8

15

TRANSPORT DATA ACROSS HIGH RESISTANCE MONOLAYERS

Figure 3 shows transport data across high resistance monolayers of bovine endothelial cells on filters in the blood-brain model of the invention, using labeled sucrose (360 dalton, hydrophilic) and
20 labeled chlorambucil (304 dalton, mildly hydrophobic anti-tumor compound).

Although these compounds are of similar size, the hydrophobic compound was transported much better than was the hydrophilic compound across filters with cells,
25 compared to transport rate across cell-free filters.

Sucrose was almost entirely prevented from leaking between the cells. This is a significant advance over previous models in which the difference in the rate of sucrose across filters with and without cells was 3 to
30 5-fold due to leaky tight junctions in those models.

EXAMPLE 9

EFFECTS OF PROTEIN KINASE INHIBITORS ON
RESISTANCE OF BRAIN ENDOTHELIAL CELLS

Bovine brain endothelial cells were grown to confluency on filters in the blood-brain model of the invention as described in Examples 1, 3 and 5 above, and the effects of protein kinase inhibitors K252a and staurosporine on resistance determined. These compounds are non-specific in that they inhibit protein kinases A and C, MLCK, etc. The results are shown in Table 6.

Table 6

<u>Drug</u>	<u>Resistance (% of control)</u>		
	<u>1 hr.</u>	<u>2.5 hr.</u>	<u>24 hr.</u>
K252a (200 nM)	79	25	24
Staurosporine			
10 nM	108	20	5.2
30 nM	64	7.7	2.3
100 nM	5.5	0	0

Both compounds, but particularly staurosporine, were remarkably effective in reducing resistance, i.e., opening up tight junctions. The effects of both inhibitors were reversible.

At the light microscope level, either removing cyclic AMP or adding a protein kinase inhibitor caused clear separation of endothelial cell tight junctions.

EXAMPLE 10

EFFECT OF PROTEIN KINASE INHIBITION
ON BRAIN UPTAKE IN VIVO

Staurosporine was administered by intracarotid infusion. The transport of ³H-sucrose and ¹²⁵I-BSA was

- 0 then determined. The data in Table 7 represent the amount of radioactivity remaining in the brain after infusion and washout. An average of 3-4 animals was used in each category.

Table 7

5	<u>Experiment 1 (DPM)</u>		
	<u>³H-Sucrose</u>	<u>¹²⁵I-BSA</u>	
	Saline control	6.2 x 10 ³	178
	Staurosporine	24.3 x 10 ³	835
10	<u>Experiment 2 (DPM)</u>		
	<u>³H-Sucrose</u>	<u>¹²⁵I-BSA</u>	
	Saline control	1.4 x 10 ³	98
	Staurosporine	8.7 x 10 ³	520

- 15 The results showed that staurosporine enhanced entry into the brain of both small (sucrose) and large (BSA) molecules, as the result of the inhibition of the activity of cyclic AMP-activated protein kinase.

EXAMPLE 11

20 EFFECT OF G_i RECEPTOR AGONISTS AND AGENTS THAT INCREASE CYCLIC GMP ON BLOOD-BRAIN BARRIER IN AN IN VIVO TEST SYSTEM

- 25 The test compound was delivered to a restrained, unanesthetized mouse (30-35g) via the tail vein. The injectate also contained 10 μ Ci ³H-sucrose and 1 μ Ci ¹²⁵I-BSA as tracer substances that do not normally penetrate the BBB in vivo. Fifteen or sixty minutes after the injection, the animal, anesthetized with 2% Avertin, was perfused via venipuncture with phosphate buffered saline followed by fixative. The brain was
30 removed immediately, the medulla, pons and hypothalamus

excised and the remaining tissue homogenized by passage through a 3 cc needle into a preweighed scintillation vial containing 1.5 ml Soluene (Packard). The vial was weighed to determine wet tissue weight. Ten ml of Inst-Gel (Packard) was added to each sample after overnight incubation at 75°C. The samples were counted for DPM by liquid scintillation spectrometry. Values are expressed as DPM/gm tissue. A minimum of four animals/group were used for each experiment. Data are expressed as the mean fold-increase which is derived by dividing the treatment value by the control value. Numbers in parenthesis represent the number of experiments carried out for the particular condition.

Table 8

15	<u>Compound</u>	<u>Radioactivity (DPM)</u>	
		<u>³H-sucrose</u>	<u>¹²⁵I-BSA</u>
	CPA, 100 ug/kg	15'	1.6 (2)
		60'	2.6 (2)
	R-PIA, 25 ug/kg	15'	2.0 (1)
		60'	3.0 (2)
20	Nitroprusside, 3 mg/kg	15'	1.5 (1)
		60'	2.3 (2)

The results show that adenosine Gi receptor agonists (such as cyclopentyladenosine, CPA) and the (-) stereoisomers of N₆-(phenylisopropyl)-adenosine (R-PIA) increased the uptake by brain of small (sucrose) and large (BSA) molecules by as much as 300%.

0

EXAMPLE 12

EFFECT OF TIGHT JUNCTION PERMEABILITY MODULATORS
ON BEHAVIORAL ASSAY

The behavioral assay is designed to demonstrate delivery of a drug into the brain parenchyma at levels sufficient to have a therapeutic effect. Morphine and the naturally occurring opioid peptides, endorphin and enkephalin, bind to μ opioid receptors in the brain and suppress the sensation of pain. This analgesic effect can be demonstrated with mice in the hot plate assay. Mice are placed on a surface uniformly heated to 55°C. The time it takes for the mouse to respond to the heat stimulus by licking its front or hind paws is measured. Morphine (700 MW) delivered by i.v. injection at doses of 1-10 mg/kg, has an analgesic effect in that it increases the latency of response to the heat stimulus measured 15 minutes after the injection. The latency is expressed as % analgesia.

$$\% \text{ analgesia} = \frac{(t_c - t_e) \times 100}{(T - t_e)}$$

t_e = experimental latency at given dose of analgesic
 t_c = control latency with no analgesic
 T = 60 sec., the maximum allowed latency

The purpose of these experiments is to test the ability of putative BBB openers to shift the morphine dose response curve to lower doses and thus have analgesic activity when delivered peripherally. In this experiment (Fig. 4) 25 μ g/kg of CPA potentiated the effects of morphine, particularly at lower morphine levels. ●, morphine alone; x, CPA + morphine

Thus, CPA, which reduces cyclic AMP production, lowered the amount of morphine that had to be administered to produce analgesia, i.e., CPA opened the blood-brain barrier.

0

EXAMPLE 13

INHIBITION BY NITROPRUSSIDE OF EFFECT OF CYCLIC AMP
ON ENDOTHELIAL CELL TIGHT JUNCTIONS

Confluent layers on filters of bovine brain endothelial cells not previously treated with cyclic AMP analogues were stimulated with RO-20-1724 (+ RO in Fig. 5) at the beginning of the experiment so as to increase cyclic AMP levels and resistance or left untreated (- RO). Other cultures were treated with RO-20-1724 plus sodium nitroprusside (NitroP) in various concentrations. Nitroprusside is known to increase the activity of the Gi system. The resistance of the cells was then determined as above.

The results, shown in Fig. 5, show that nitroprusside inhibited the increase in resistance produced by cyclic AMP elevation in a dose-dependent manner.

EXAMPLE 14

EFFECT OF SODIUM NITROPRUSSIDE ON
MORPHINE-INDUCED ANALGESIA

The effects of sodium nitroprusside, an agent that increases cyclic GMP levels, on morphine-induced analgesia in mice were determined by the assay system of Example 12, and are shown in Fig. 6. Nitroprusside (+ NP) decreased the amount of morphine required to produce analgesia, suggesting that the former opened up the blood-brain barrier to the latter.

EXAMPLE 15

APPEARANCE OF E-CADHERIN ON ENDOTHELIAL CELLS

Bovine brain endothelial cells were grown to confluency on filters either in control medium (low resistance cultures) or under conditions of enhanced

0 cyclic AMP plus endothelial cell conditioned medium as
in Examples 1, 3 and 5 (high resistance cultures).
After transendothelial cell resistance measurements,
cultures were fixed and labeled with a rabbit antibody
prepared against mouse E-cadherin with a fluorescent
5 FITC-conjugate of goat anti-rabbit immunoglobulin. The
high resistance cultures stained much more brightly for
E-cadherin than did low resistance cultures, and also
showed some localization of the E-cadherin around cell
borders. As E-cadherin appears to be expressed
10 specifically in endothelial cells in brain, these
observations are a further demonstration that
treatments that increase electrical resistance of brain
endothelial cells in culture also cause them to adopt
another characteristic property of the BBB.

15

EXAMPLE 16

EFFECT OF INHIBITION OF CYCLIC GMP
PHOSPHODIESTERASE ON ENKEPHALIN-INDUCED ANALGESIA

The behavioral assay described in Example 12,
modified to induce analgesia with an endogenous opiate,
20 enkephalin (20 mg/kg), rather than with morphine, was
applied to mice treated with the cyclic GMP
phosphodiesterase inhibitor, dipyridamole. The results
are shown in Table 9.

0

Table 9

<u>Experiment</u>	<u>Percent Analgesia</u>	
	<u>Enkephalin</u>	<u>Enkephalin + Dipyridamole</u>
1	2.8	13.5
2	6.4	25.8

5

These results are consistent with the theory that elevation of intracellular levels of cyclic GMP increase the permeability of brain microvascular endothelial cell tight junctions to morphine.

10

EXAMPLE 17

EFFECT OF INHIBITION OF CYCLIC GMP PHOSPHODIESTERASE
ON BLOOD-BRAIN BARRIER IN AN IN VIVO TEST SYSTEM

The in vivo tracer experiment of Example 11 was repeated, except that the test compound was the cyclic GMP phosphodiesterase inhibitor, dipyridamole.

15

Table 10

<u>Condition</u>	<u>Time min.</u>	<u>DPM/gm tissue</u>	
		<u>³H-sucrose</u>	<u>¹²⁵I-BSA</u>
Control	15	8,757 ± 2034	616 ± 46
	15	18,233 ± 4770	1110 ± 187
Dipyridamole	60	12,164 ± 1774	579 ± 47
	60	17,932 ± 2642	943 ± 157

25

The results show that inhibition of cyclic GMP phosphodiesterase, which results in an elevation of intracellular levels of cyclic GMP, increased the

- 0 transport across the blood-brain barrier of both small
(sucrose) and large (bovine serum albumin) molecules.

EXAMPLE 18

EFFECTS OF DIFFERENT CLASSES OF PHOSPHODIESTERASE INHIBITORS ON BRAIN

5 MICROVASCULAR ENDOTHELIAL CELL TIGHT JUNCTIONS IN VITRO

Bovine brain microvascular endothelial cells were isolated and grown on permeable filters in the BBB in vitro model of the invention. Cells were maintained in astrocyte-derived conditioned medium, but were not
10 treated with agents that elevate cyclic AMP levels in such cells.

At the beginning of the experiment (i.e., in a low resistance state), cells were either left untreated (blk in Figure 7) or treated with 17.5 μ M Rolipram or
15 RO-20-1724 (RO in Figure 7) (specific inhibitors of cyclic AMP phosphodiesterase). Resistance was then measured at various times. In both experiments (Figure 7), by 30 minutes resistance was already substantially higher in cells treated with these compounds. In
20 contrast, specific inhibitors of cyclic GMP phosphodiesterase, zaprinast, dipyridamole and milrinone, were ineffective under analogous conditions. This suggests that the phosphodiesterase predominantly responsible for degrading cyclic AMP in brain
25 endothelial cells is a Type III cyclic GMP-noninhibitable phosphodiesterase. This also suggests that inhibitors of this class of enzyme may be effective in treating vasogenic brain edema.

0

EXAMPLE 19

RESISTANCE OF HUMAN BRAIN MICROVASCULAR ENDOTHELIAL
CELLS IN AN IN VITRO BLOOD-BRAIN BARRIER MODEL

Brain biopsy specimens from human epileptic patients were transported from the operating room to the laboratory in a MEM-antibiotic medium. After dissecting away the meninges, gray matter was rinsed, then homogenized in L-15 medium as described above. The homogenate was passed through a 50 μ m nylon filter; the residue was passed through a 50 μ m filter twice more. The final residue was spun down, then suspended in 5 ml of a solution containing collagenase, trypsin and DNase, as described above for bovine brain endothelial cells. Isolated capillary fragments and cells were centrifuged, resuspended and plated on collagen-fibronectin-coated flasks in a growth medium containing rat astrocyte-derived conditioned medium. The cells were then maintained as described above for bovine brain cells.

After the cells had been transferred to permeable supports and grown to confluency, some were treated with chlorophenyl-thio-cyclic AMP + RO-20-1724 as detailed above.

Prior to elevation of cyclic AMP levels, resistance of the cells (average of six replicates) was 62.5 ohm-cm². Subsequent to treatment, resistance of the cells (average of six replicates) averaged 357.8 ohm-cm².

This experiment demonstrated that human brain microvascular endothelial cells responded as did the counterpart cells from bovine brain in terms of tight junction formation in response to cyclic AMP.

0

EXAMPLE 20

EFFECT OF REMOVAL OF CYCLIC
AMP-ELEVATED AGENTS ON THE SUBSEQUENT RESISTANCE
OF BRAIN MICROVASCULAR ENDOTHELIAL CELLS

5 Bovine brain endothelial cells were grown to
 10 confluency on filters in the blood-brain barrier of the
 invention using the standard protocol described in
 Examples 1, 3 and 5. Thereafter, cells were treated
 for 48 hours with chlorophenyl-thio-cyclic AMP +
 RO-20-1724. The medium containing these cyclic AMP
 15 elevating agents was removed, and the cells were washed
 with fresh growth medium. To some cells was added
 growth medium alone, and to other cells was added
 growth medium containing the cyclic AMP analogue and
 RO-20-1724. The resistance of the cell monolayers was
 20 measured periodically, and the results are shown in
 Table 11.

Table 11

		<u>Resistance</u>	
20	<u>Time of</u> <u>second incubation</u> <u>min.</u>	<u>Medium alone</u>	<u>Medium +</u>
		<u>ohm-cm²</u>	<u>"cyclic AMP"</u> <u>(average)</u>
	Initial	322	322
	15	74	212
	30	69	284
25	60	59	293
	Overnight	39	332

Resistance dropped quite rapidly when
 intracellular levels of cyclic AMP were reduced (Medium
 30 alone in Table 11), due, probably, to the presence of a
 rapidly acting phosphoprotein phosphatase that

- 0 dephosphorylates cyclic AMP-activated proteins.
Resistance remained high when washed cells continued to
be exposed to sources of intracellular cyclic AMP
(Medium + "cyclic AMP").

EXAMPLE 21

5 MODULATION OF LEUKOCYTE ADHESION TO
INFLAMED BRAIN ENDOTHELIAL CELLS

In this preferred embodiment, antibodies against
VLA-4 were shown to substantially prevent leukocyte
adhesion to brain endothelial, using both a novel
10 system for inducing MS-type inflammation in vivo, and
the blood brain barrier model.

Unless otherwise stated, all technical and
scientific terms used herein have the same meaning as
commonly understood by one of ordinary skill in the art
15 to which this invention belongs. Although any methods
and materials similar or equivalent to those described
herein can be used in the practice or testing of the
present invention, the preferred methods and materials
are now described. As set forth above, all
20 publications to which reference is made are
incorporated herein by reference.

Materials and Methods

Lymphocytes: Mouse or rat lymphocytes were
isolated immediately before a binding assay from
25 mesenteric, cervical and brachial lymph nodes by
standard methods and crushed between the frosted ends
of two glass slides. Human lymphocytes were isolated
from heparinized or EDTA-treated whole blood using
Mono/Poly separation medium (Flow Labs, Mclean, VA),
30 and used immediately.

Lymphoid Cell Lines: All cell lines were obtained
from the cell culture facility at the University of

0 California, San Francisco and were maintained in RPMI-
1640 with 10% FBS (37°C, 10% CO₂). RPMI-1460 was
purchased from the University of California, San
Francisco. These University of California cell lines
are all made available to the public. Specifically, the
5 cell lines obtained from the University of California,
San Francisco are the Jurkat T-cell line, U937, THP-1,
FRO, HL60, and HUT78. These cell lines may also be
available from other sources.

Monoclonal Antibodies: AIIB2, against human β 1
10 integrin (the "anti- β -1"), is available from Dr.
Caroline Damsky, Department of Oral Biology, University
of California, San Francisco. HP2/1, against the VCAM-
1-binding domain of VLA-4 (the "anti- α -4") was pur-
chased from AMAC, Inc. (Westbrook ME, Product # 0764).
15 The AP2/1 also cross-reacts with murine lymphocytes.
P4H9, against human β 2 integrin (the "anti- β -2") was
purchased from Telios, Inc. (San Diego, CA Product
#A052). This anti- β -2, against the β -2 integrin is not
known to react with any subunit of VLA-4.

20 When used to treat lymphocytes, the anti- β -1
hybridoma supernatant was used at a 1:2 dilution. The
anti- α -4 antibody was purified by the manufacturer, and
used at a 5 μ g/ml concentration. The anti- β -2 was
purified by the manufacturer, and used at 5 μ g/ml. For
25 treatment of lymphocytes, the lymphocytes were mixed
with the above concentrations of antibody, and allowed
to incubate on ice for a about 30 minutes prior to use.
The cells were washed to remove unbound antibody, and
resuspended in RPMI to a typical concentration of 10⁷
30 cells/ml.

Other tissue sections: In the in vitro frozen
brain section assay, the preparation of brain sections
is described below. Lymph node and intestinal tissues

0 were removed from rats, and sectioned as described for
brain tissues, below.

A. In Vitro Frozen Brain Section Assay

In order to establish inflammatory brain lesions
that involve a large degree of immune cell
5 infiltration, rats were injected in the brain with
human kidney cell line 293 (American Type Culture
Collection, "ATCC," 1573). This method was found to
stimulate the entry of all leukocyte classes into the
brain in a predictable time course. The trauma of the
10 injection induces the entry of neutrophils and
monocytes within minutes, which continues for about 24
to about 48 hours. The presence of the human cells
serves as a persistent irritant to the immune system,
stimulating further leukocyte infiltration, including
15 that of lymphocytes. Typically, by about day 6,
lymphocytes and monocytes are the major infiltrating
leukocyte classes, entering in such large numbers so as
to produce cellular cuffs around small blood vessels in
the brain near the injection site. The speed and
20 predictability of this procedure has made it ideal for
obtaining brain tissue that can be used in the in vitro
assay described below. In this assay, the brains are
quickly frozen and sectioned. Leukocytes (immortalized
and grown as cell lines, or freshly isolated from
25 rodents or humans as described above) are then exposed
to the sections, and, if they express the appropriate
receptors, adhere selectively to the exposed profiles
of activated endothelium near the inflammatory lesion.
The leukocytes do not bind to nonactivated endothelium
30 in the brain sections away from the inflammatory lesion
or within sections of the non-stimulated control brain.

Rats (male Sprague-Dawley, 275-300g), were
anesthetized with Nembutal (60 mg/kg-i.p.) and mounted

0 in a stereotaxic device. The head was shaved and an
incision was made to reveal the dorsal skull. Holes
were drilled through the skull on the left and right
side overlying the parietal cortex. 10^7 human kidney
derived cells (ATCC 1573 cell line), suspended in PBS,
5 were delivered to the parietal cortices in a volume of
 $10\mu\text{l}$. It is believed that other allogeneic cells or
cell lines would also induce the present MS-type
symptoms via these methods. For example, we have used
primary bovine microvascular endothelial cells to
10 induce a similar inflammatory reaction.

The incision was sutured and the animal allowed to
recover for 1-10 days. On the appropriate day, brains
were removed from animals that had been anesthetized
with halothane and killed by cardiac puncture. The
15 cerebellum was removed and the brains were then placed
rostral side down on a mound of gum tragacanth (mixed
with water to the consistency of thick paste) and
frozen by immersion for 60 seconds in 2-methyl butane
chilled on dry ice. The brains were then stored in
20 sealed tubes at -80°C .

Immediately before the assay, 10 micron thick
brain sections were cut on a cryostat and transferred
to the center of a 14 mm well, pre-formed within a thin
epoxy coating (Catalog #100314, Carlson Scientific,
25 Inc., Peotone, IL) and allowed to air-dry at room
temperature. The transfer was accomplished by touching
the slide (at room temperature) to the section, which
was still on the cold knife blade. Sections of control
tissues (peripheral lymph nodes and Peyer's patches,
30 isolated from noninjected rats, frozen and stored as
described above) were usually placed adjacent to the
brain sections in the same wells. The slides were
positioned on a metal tray resting on ice and the wells

0 were filled with 100 μ l of the appropriate cell
suspension. The metal tray and supporting ice were
then gyrated at about 50- about 80 rpm for 30 minutes
on an orbital shaker (Lab Line Instruments, Inc., Model
3520, with 1 inch diameter rotation). The cell
5 suspension was then decanted and the slides were
carefully placed vertically in PBS with 2.5%
glutaraldehyde on ice for 20 minutes. The slides were
then dipped 5 times in PBS, placed in 0.5% toluidine
blue (20% EtOH) for 1 minutes, destained with 2 brief
10 dips in 100% ethanol, covered with Immu-mount™
mounting medium (Shandon, Sweickley, PA), and cover
slipped.

Herein, cell suspensions used were freshly
isolated rat, mouse or human lymphocytes, the U937T
15 human myelomonocytic cell line, and the Jurket human T
cell line. Cell lines THP-1, FRO, HL60 and HUT78 were
found not to bind with stimulated brain sections, and
were not further analyzed in the brain section assay.

The degree of lymphocyte binding was quantified by
20 one of two methods. The first relied upon an internal
reference population of cells, similar to that
described by Butcher, et al., J.Immunol 123: 1996-2003
(1979). The second method was based on the absolute
number of lymphocytes bound to blood vessels in a given
25 tissue section. For the internal reference method,
populations of lymphoid cell lines were mixed with
freshly isolated lymphocytes of a different species
(e.g. human cell lines with mouse lymphocytes) so that
both were at a final concentration of $3-5 \times 10^7$ /ml.
30 Aliquots of the mixed population were then treated with
species-specific antibodies for 30 minutes on ice. In
general, the cells were washed out of the antibody
prior to the binding assay. Binding was quantified by

0 determining the ratio of two different populations of
 leukocytes bound to blood vessels. It was always
 arranged such that the leukocyte populations could
 readily be distinguished by size--lymphoid cell lines
 are large cells, generally greater than 20 μm in
 5 diameter, while lymphocytes are small cells, less than
 10 μm in diameter. Thus in an experiment with human T
 cell line mixed with rat lymphocytes, the degree of
 inhibition produced by an anti-human monoclonal
 antibody (compared to control antibodies or to no
 10 treatment) was quantified by determining the ratio of
 large to small cells bound. The results obtained are
 presented in Table 12, below. As can be seen, use of
 an anti-VLA-4 reagent significantly inhibited binding
 of immune cells to brain cells displaying MS-type
 15 inflammation. These results are also shown in Figure
 10, which clearly displays the inhibitory effect of the
 anti-VLA-4 reagents.

Table 12

20 Quantification of rat lymphocyte binding to different
 tissue sections by comparing the number of lymphocytes
 bound to all vessels under treated and untreated
 conditions. Four replicates were used for each
 treatment, and raw data are presented in parenthesis
 below the mean.

25	<u>Treatment</u>	<u>Brain</u>	<u>Intestinal</u>	<u>Lymph Node</u>
	No treatment	24(100%)	43(100%)	21(100%)
		(24/24/32/18)	(35/45/42/50)	(25/27/18/15)
	anti-VLA- α	2(8%)	6(15%)	20(95%)
		(2/3/2/2)	(3/3/5/10)	(14/30/22/12)

0 The second method of quantification compared a
 single population of leukocytes treated in different
 ways in adjacent assay wells. The degree of inhibition
 was determined by comparing the number of leukocytes
 bound to all vessels in a given tissue section under
 5 treated and untreated conditions. Tissue sections were
 prepared as described above. The degree of binding was
 quantified as the actual number of cells bound to the
 blood vessels within the sections. These data are
 presented in Table 13, below.

10 In addition, lymph node tissue was also tested,
 and these data are displayed in Figure 11. All
 reagents were prepared as described above. Here, anti-
 β -1 antibody and anti- α -4 antibody were both shown to
 inhibit Jurkat T-cell lymphocyte binding to brain
 15 sections, but not to lymph node sections.

These data confirm that anti-VLA-4 reagents showed
 substantial inhibition of leukocyte binding to brain
 tissue displaying the features of MS-type inflammation.

Table 13

20 <u>Treatment Used</u>	<u>Ratio large/small</u>	<u>% Control Jurkat Binding</u>
No treatment	2.08 ± 0.17	100 ± 8
anti- β -1	0.02 ± 0.01	1 ± 0.5
anti- α -4	0.23 ± 0.15	11 ± 7

25 B. Leukocyte Binding to Cultures of Blood Brain
 Barrier Endothelial Cells

Bovine or human brain endothelial cells were
 maintained in accordance with the blood brain barrier
 model as described in the present specification. In

0 experiments where the endothelium was activated, 5 μ l
of stimulating agent was added directly to the lower
chamber medium (800 μ l) of the culture system. Here,
TNF α (Amgen Biologicals, Thousand Oaks, CA) was added
to the lower chamber for a final concentration of 400
5 μ /ml. In activation, PMA-S (in DMSO) was found not to
be effective in stimulating lymphocyte binding, but
other activating agents are known, and will be apparent
to those skilled in the art.

In this manner, the endothelial cells were exposed
10 to the agent on their ablumenal surface, as would be
the typical situation during an inflammatory reaction
in the brain. Immediately before the assay, the
electrical resistance of the cultures was measured and
the filters (supporting the endothelial cells) were
15 washed at room temperature by dipping in three separate
vats of D-MEM with 1% FBS and 20 mM Hepes (200 mls.
each). The filters were then placed in fresh wells
containing the same medium and the assay was performed
at room temperature.

20 Typically, 10 μ l of leukocytes (at a preferred
concentration of 10^7 /ml) in the presence or absence of
test reagents, were added to the upper chamber of the
culture system, such that the leukocytes would
encounter the luminal or blood side of the endothelium,
25 as they would in the brain vasculature. Here,
leukocytes were rat, mouse or human lymphocytes, the
U937 human myelomonocytic cell line, and the Jurket
human T cell line as described above. The lymphocytes
were pretreated with anti- β -1 or anti- β -2 antibody as
30 described above.

Cell lines THP-1 and FRO were also found to bind
to brain endothelial stimulated with TNF α , but HL60 and

0 HUT78 did not so bind. U937 binding was found not to be inhibited by exposure to anti- β -1.

The culture plates were placed on a gyratory shaker at about 100 rpm for 30 seconds, then allowed to sit undisturbed at room temperature for about 30
5 minutes. The assay was terminated by gently washing the filters in PBS with 1% glutaraldehyde (dipping and pouring three times at different angles). The glutaraldehyde causes the cells to fluoresce under the proper optical conditions, as described below. The
10 filters were then allowed to fix in the glutaraldehyde solution undisturbed for 60 minutes.

The degree of leukocyte binding to the filters was examined in one of two ways. In the first, the bound cells were visualized directly. The filter was cut
15 free of the culture well apparatus and mounting on glass slides with Immu-mountTM. The filters were examined with an immunofluorescence microscope set for rhodamine or fluorescein optics, and observing the cells by glutaraldehyde-induced autofluorescence.

20 The results of the immunofluorescence assay can be visualized in Figure 8. As is easily visualized, the density of Jurkat T-cell lymphocytes pretreated with anti- β -1 antibody is far lower (Panel A) than the binding density for untreated leukocytes (Panel B).
25 This graphically depicts the anatomical and physiological reaction when a reagent is used to block the VCAM-1/VLA-4 interaction between brain endothelial cells and leukocytes.

In the second method, the leukocytes were pre-
30 labeled with a radioactive tracer and the degree of binding was quantified by measuring the amount of radioactivity associated with the entire endothelial surface on the culture filter. Prelabeling of lymphoid

0 cell lines was accomplished by the addition of 1 uCi/ml
125IUDR (Amersham #XX) obtained from the Amersham
Corporation, Arlington Heights, Illinois, to the
standard culture medium approximately 12-20 hrs. before
the assay. The cells were washed free of
5 unincorporated label by three separate washes in 15 mls
of fresh bench medium (RPMI-1640 with 5% FBS and 25 mM
Hepes). Concentration was then adjusted to 10^7 cells
/ml in the presence or absence of test reagents. Again,
all of the above lymphocytes were used (rat, mouse or
10 human lymphocytes, the U937T human myelomonocytic cell
line, and the Jurket human T cell line) as described
above. The lymphocytes were pretreated with anti- β -1
or anti- β -2 antibody as described above. Also, the
lymphocytes were pretreated with anti-VLA- α -4 as
15 described above.

The assay was carried out as above, except that at
the end the isolated filters are placed in tubes and
counted in a gamma counter (Beckman Corporation, Model
5500B) for 1 minute. The results are presented in
20 Table 14 below. As can be seen, the samples that
contained anti-VLA-4 reagents show far lower
radioactivity levels the controls. These data confirm
the results from the above fluorescence data, namely,
that the binding of anti-VLA-4 to the VCAM-1 receptor
25 is substantially inhibited by reagents which would
prevent binding at that locus.

These data are also presented at Figure 11 which
shows the relative degree of Jurkat T-cell lymphocyte
binding to brain endothelial cells in the BBB system.
30 As can easily be seen, the anti- β -1 antibody
effectively inhibited the binding of leukocytes to TNF-
 α activated brain endothelial cells. Anti- β -2, as a
control, on the other hand, approaches the untreated

- 0 control. Plainly, the β -1 subunit provides an effective target for preventing VLA-4/VCAM-1 interaction in the brain.

Table 14

5 This table shows the quantification of lymphocyte binding via radioactive labelling of lymphocytes.

	<u>Lymphocyte Type</u>	<u>Treatment</u>	<u>Count per Minute</u>
	Jurkat T-cell		
10		0 (no TNF α)	112; 273
		anti- β -1 (no TNF α)	259; 156
		anti- β -2 (no TNF α)	198; 124
		0 (+ TNF α)	1430; 1150
		anti- β -1 (+ TNF α)	361; 385
15		anti- β -2 (+ TNF α)	1313; (n/a)

The above discussion of this invention is directed primarily to preferred embodiments and practices thereof. It will be readily apparent to those skilled in the art that further changes and modifications in
20 the actual implementation of the concepts described herein can easily be made without departing from the spirit and scope of the invention as defined by the following claims.

We claim:

1. An in vitro model of a blood-brain barrier, comprising a porous solid support upon which is disposed an essentially confluent monolayer of brain microvascular endothelial cells and a means for augmenting cyclic AMP concentrations in said cells such that peripheral phalloidin staining is substantially present.
2. A model as recited in claim 1, wherein said endothelial cells are mixed endothelial cells.
3. A model as recited in claim 1, wherein said porous solid support is a porous filter or membrane of a material selected from the group consisting of polycarbonate, nitrocellulose, cellulose, collagen and fiberglass.
4. A model as recited in claim 1, further comprising a first coat of an extracellular matrix material disposed upon said porous solid support interposed between a second coat of said endothelial cells and said porous solid support.
5. A model as recited in claim 4, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel[®].
6. A model as recited in claim 1, wherein said means for augmenting effective endothelial cell cyclic AMP concentration is selected from among the group consisting of agents that increase cell adenylate cyclase activity, agents that inhibit the degradation of intracellular cyclic AMP, and derivatives of cyclic AMP to which endothelial cells are permeable and which act physiologically as does cyclic AMP.

7. An in vitro model of a blood-brain barrier as recited in claim 1, further comprising an essentially confluent monolayer of brain astrocytes disposed on a side of said porous solid support opposite the side on which are disposed said endothelial cells.

8. An in vitro model of a blood-brain barrier as recited in claim 1, further comprising an essentially confluent monolayer of brain astrocytes disposed on a second surface other than the porous solid support upon which is disposed a monolayer of endothelial cells, in contact with a growth medium to which the endothelial cells are also exposed.

9. A model as recited in claim 1, wherein said porous solid support comprises microcarrier beads.

10. A model as recited in claim 9, further comprising a first coat of an extracellular matrix material disposed on said beads, interposed between said beads and said endothelial cells.

11. A model as recited in claim 10, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel[®].

12. A model as recited in claim 1, wherein said porous solid support comprises a tubular hollow fiber.

13. A model as recited in claim 12, further comprising a first coat of an extracellular matrix material disposed on said hollow fiber, interposed between said hollow fiber and said endothelial cells.

14. A model as recited in claim 13, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte

extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel^R.

15. A model as recited in claim 1, wherein E-cadherin is substantially present between endothelial cells.

16. An in vitro model of a blood-brain barrier, comprising a porous solid support upon which is disposed an essentially confluent monolayer of brain microvascular endothelial cells and a means for augmenting effective cyclic AMP in such cells, such that the transmonolayer resistance is at least about 200 ohm-cm².

17. A model as recited in claim 16, wherein said endothelial cells are mixed or cloned endothelial cells.

18. A model as recited in claim 16, wherein said porous solid support is a porous filter or membrane selected from the group consisting of polycarbonate, nitrocellulose, cellulose, collagen and fiberglass.

19. A model as recited in claim 16, further comprising a first coat of an extracellular matrix material disposed upon said porous solid support interposed between said endothelial cells and said porous solid support.

20. A model as recited in claim 19, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel^R.

21. A model as recited in claim 16, wherein said means for augmenting effective endothelial cell cyclic AMP concentration is selected from among the group consisting of agents that increase cell adenylate

cyclase activity, agents that inhibit the degradation of intracellular cyclic AMP, and derivatives of cyclic AMP to which endothelial cells are permeable and which act physiologically as does cyclic AMP.

22. An in vitro model of a blood-brain barrier as recited in claim 16, further comprising an essentially confluent monolayer of brain astrocytes disposed on a side of said porous solid support opposite the side on which are disposed said endothelial cells.

23. An in vitro model of a blood-brain barrier as recited in claim 16, further comprising an essentially confluent monolayer of brain astrocytes disposed on a second surface other than the porous solid support upon which is disposed a monolayer of endothelial cells, in contact with a growth medium to which the endothelial cells are also exposed.

24. A model as recited in claim 16, wherein said porous solid support comprises a tubular hollow fiber.

25. A model as recited in claim 24, further comprising a first coat of an extracellular matrix material disposed on said hollow fiber, interposed between said hollow fiber and said endothelial cells.

26. A model as recited in claim 25, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel^R.

27. A model as recited in claim 16, wherein E-cadherin is substantially present between endothelial cells.

28. An in vitro model of a blood-brain barrier comprising a porous solid support upon which is

disposed an essentially confluent monolayer of brain microvascular endothelial cells, a means for augmenting effective cyclic AMP concentrations in said cells, and a means for growing the brain microvascular endothelial cells in a cell-derived conditioned medium or the equivalent, such that peripheral phalloidin staining is substantially present.

29. A model as recited in claim 28, wherein said endothelial cells are mixed or cloned endothelial cells.

30. A model as recited in claim 28, wherein said porous solid support is a porous filter or membrane selected from among the group consisting of polycarbonate, nitrocellulose, cellulose, collagen and fiberglass.

31. A model as recited in claim 28, further comprising a first coat of an extracellular matrix material disposed upon said porous solid support interposed between said endothelial cells and said porous solid support.

32. A model as recited in claim 31, wherein said first coat extracellular matrix is selected from among the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel[®].

33. A model as recited in claim 28, wherein said means for augmenting effective endothelial cell cyclic AMP concentration is selected from among the group consisting of agents that increase cell adenylate cyclase activity, agents that inhibit the degradation of intracellular cyclic AMP, and derivatives of cyclic AMP to which endothelial cells are permeable and which act physiologically as does cyclic AMP.

34. A model as recited in claim 28 wherein said cell-derived conditioned medium comprises endothelial cell-derived conditioned medium or astrocyte-derived conditioned medium, and said equivalent comprises a tissue extract.

35. An in vitro model of a blood-brain barrier as recited in claim 28, further comprising an essentially confluent monolayer of brain astrocytes disposed on a side of said porous solid support opposite the side on which are disposed said endothelial cells.

36. An in vitro model of a blood-brain barrier as recited in claim 28, further comprising an essentially confluent monolayer of brain astrocytes disposed on a second surface other than the porous solid support upon which is disposed a monolayer of endothelial cells, in contact with a growth medium to which the endothelial cells are also exposed.

37. A model as recited in claim 28, wherein said porous solid support comprises microcarrier beads.

38. A model as recited in claim 37, further comprising a first coat of an extracellular matrix material disposed on said beads, interposed between said beads and said endothelial cells.

39. A model as recited in claim 38, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel[®].

40. A model as recited in claim 28, wherein said porous solid support comprises a tubular hollow fiber.

41. A model as recited in claim 40, further comprising a first coat of an extracellular matrix

material disposed on said hollow fiber, interposed between said hollow fiber and said endothelial cells.

42. A model as recited in claim 41, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel[®].

43. A model as recited in claim 28, wherein E-cadherin is substantially present between endothelial cells.

44. An in vitro model of a blood brain barrier comprising a porous solid support upon which is disposed an essentially confluent monolayer of brain microvascular endothelial cells, a means for augmenting effective cyclic AMP concentrations in said cells and a means for growing said endothelial cells in a cell-derived conditioned medium or the equivalent, such that peripheral phalloidin staining is substantially present, and having a trans-monolayer resistance of at least about 200 ohm-cm².

45. A model as recited in claim 44, wherein said endothelial cells are mixed or cloned endothelial cells.

46. A model as recited in claim 44, wherein said porous solid support is a porous filter or membrane selected from among the group consisting of polycarbonate, nitrocellulose, cellulose, collagen and fiberglass.

47. A model as recited in claim 44, further comprising a first coat of an extracellular matrix material disposed upon said porous solid support interposed between said endothelial cells and said porous solid support.

48. A model as recited in claim 47, wherein said first coat extracellular matrix material is selected from among the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel^R.

49. A model as recited in claim 44, wherein said means for augmenting effective endothelial cell cyclic AMP concentration is selected from among the group consisting of agents that increase cell adenylate cyclase activity, agents that inhibit the degradation of intracellular cyclic AMP, and derivatives of cyclic AMP to which endothelial cells are permeable and which act physiologically as does cyclic AMP.

50. A model as recited in claim 44, wherein said cell-derived conditioned medium comprises endothelial cell-derived or astrocyte-derived conditioned medium, and said equivalent comprises a cell extract.

51. An in vitro model of a blood-brain barrier as recited in claim 44, further comprising an essentially confluent monolayer of brain astrocytes disposed on a side of said porous solid support opposite the side on which are disposed said endothelial cells.

52. An in vitro model of a blood-brain barrier as recited in claim 44, further comprising an essentially confluent monolayer of brain astrocytes disposed on a second surface other than the porous solid support upon which is disposed a monolayer of endothelial cells, in contact with a growth medium to which the endothelial cells are also exposed.

53. A model as recited in claim 44, wherein said porous solid support comprises a tubular hollow fiber.

54. A model as recited in claim 53, further comprising a first coat of an extracellular matrix material disposed on said hollow fiber, interposed between said hollow fiber and said endothelial cells.

55. A model as recited in claim 54, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel[®].

56. A model as recited in claim 44, wherein E-cadherin is substantially present between endothelial cells.

57. An in vitro model of a blood-brain barrier, comprising a porous solid support upon which is disposed an essentially confluent monolayer of brain microvascular endothelial cells and a means for augmenting effective cyclic AMP concentrations in said cells such that peripheral phalloidin staining is substantially present, and having a transmonolayer electric resistance of at least about 200 ohm-cm².

58. A model as recited in claim 57, wherein said endothelial cells are mixed endothelial cells.

59. A model as recited in claim 57, wherein said porous solid support is a porous filter or membrane selected from the group consisting of polycarbonate, nitrocellulose, cellulose, collagen and fiberglass.

60. A model as recited in claim 57, further comprising a first coat of an extracellular matrix material disposed upon said porous solid support interposed between a second coat of said endothelial cells and said porous solid support.

61. A model as recited in claim 60, wherein said first coat extracellular matrix material is

selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel[®].

62. A model as recited in claim 57, wherein said means for augmenting effective endothelial cell cyclic AMP concentration is selected from among the group consisting of agents that increase cell adenylate cyclase activity, agents that inhibit the degradation of intracellular cyclic AMP, and derivatives of cyclic AMP to which endothelial cells are permeable and which act physiologically as does cyclic AMP.

63. An in vitro model of a blood-brain barrier as recited in claim 57, further comprising an essentially confluent monolayer of brain astrocytes disposed on a side of said porous solid support opposite the side on which are disposed said endothelial cells.

64. An in vitro model of a blood-brain barrier as recited in claim 57, further comprising an essentially confluent monolayer of brain astrocytes disposed on a second surface other than the porous solid support upon which is disposed a monolayer of endothelial cells, in contact with a growth medium to which the endothelial cells, in contact with a growth medium to which the endothelial cells are also exposed.

65. A model as recited in claim 57, wherein said porous solid support comprises a tubular hollow fiber.

66. A model as recited in claim 65, further comprising a first coat of an extracellular matrix material disposed on said hollow fiber, interposed between said hollow fiber and said endothelial cells.

67. A model as recited in claim 66, wherein said first coat extracellular matrix material is

selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel^R.

68. A model as recited in claim 57, wherein E-cadherin is substantially present between endothelial cells.

69. An in vitro model of a blood-brain barrier, comprising a porous solid support upon which is disposed an essentially confluent monolayer of brain microvascular endothelial cells, a means for augmenting effective cyclic AMP concentrations in said endothelial cells, and a means for growing said cells in a cell-derived conditioned medium or the equivalent, such that the transmonolayer electrical resistance is at least about 200 ohm-cm².

70. A model as recited in claim 69, wherein said endothelial cells are mixed or cloned endothelial cells.

71. A model as recited in claim 69, wherein said porous solid support is a porous filter or membrane selected from the group consisting of polycarbonate, nitrocellulose, cellulose, collagen and fiberglass.

72. A model as recited in claim 69, further comprising a first coat of an extracellular matrix material disposed upon said porous solid support interposed between a second coat of said endothelial cells and said porous solid support.

73. A model as recited in claim 72, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel^R.

74. A model as recited in claim 69, wherein said means for augmenting effective cyclic AMP concentration in said endothelial cells is selected from among the group consisting of agents that increase cell adenylate cyclase activity, agents that inhibit the degradation of intracellular cyclic AMP, and derivatives of cyclic AMP to which endothelial cells are permeable and which act physiologically as does cyclic AMP.

75. A model as recited in claim 69, wherein said cell-derived conditioned medium comprises endothelial cell-derived or astrocyte-derived conditioned medium, and said equivalent comprises a tissue extract.

76. An in vitro model of a blood-brain barrier as recited in claim 69, further comprising an essentially confluent monolayer of brain astrocytes disposed on a side of said porous solid support opposite the side on which are disposed said endothelial cells.

77. An in vitro model of a blood-brain barrier as recited in claim 69, further comprising an essentially confluent monolayer of brain astrocytes disposed on a second surface other than the porous solid support upon which is disposed a monolayer of endothelial cells, in contact with a growth medium to which the endothelial cells are also exposed.

78. A model as recited in claim 69, wherein said porous solid support comprises a tubular hollow fiber.

79. A model as recited in claim 78, further comprising a first coat of an extracellular matrix material disposed on said hollow fiber, interposed between said hollow fiber and said endothelial cells.

80. A model as recited in claim 79, wherein said first coat extracellular matrix material is selected from the group consisting of astrocyte extracellular matrix, laminin, vitronectin, fibronectin, collagen, and Matrigel^R.

81. A model as recited in claim 69, wherein E-cadherin is substantially present between endothelial cells.

82. A model as recited in claim 1, wherein said microvascular endothelial cells are brain capillary cells.

83. A model as recited in claim 16, wherein said microvascular endothelial cells are brain capillary cells.

84. A model as recited in claim 28, wherein said microvascular endothelial cells are brain capillary cells.

85. A model as recited in claim 44, wherein said microvascular endothelial cells are brain capillary cells.

86. A model as recited in claim 57, wherein said microvascular endothelial cells are brain capillary cells.

87. A model as recited in claim 69, wherein said microvascular endothelial cells are brain capillary cells.

88. A drug delivery composition for opening tight junctions between microvascular endothelial cells, whereby means are provided for said drug to cross the permeability barrier created by such junctions, comprising an agent capable of reducing the effective intracellular concentration of cyclic AMP, so that cell-cell adhesion is disrupted.

89. The composition of claim 88, wherein said cells are brain microvascular endothelial cells and said permeability barrier is the blood-brain barrier.

90. The composition of claim 88, wherein said agent is selected from among the group consisting of agents that reduce adenylate cyclase activity, agents that inhibit the functioning of G_s agonists, agents that stimulate the degradation of cyclic AMP or cyclic AMP analogues or derivatives, and agents that inhibit the penetration of cells by cyclic AMP or analogues or derivatives of cyclic AMP.

91. A drug delivery composition for opening tight junctions between microvascular endothelial cells, whereby means are provided for a drug to cross the permeability barrier created by such junctions, comprising an agent capable of reducing the physiological effects of cyclic AMP, so that cell-cell adhesion is disrupted.

92. The composition of claim 91, wherein said cells are brain microvascular endothelial cells and said permeability barrier is the blood-brain barrier.

93. The composition of claim 91, wherein said agent is an agent that inhibits cyclic AMP-activatable protein kinase or stimulates a phosphoprotein phosphatase.

94. A drug delivery composition for opening tight junctions between microvascular endothelial cells, whereby means are provided for a drug to cross the permeability barrier created by such functions, comprising an agent capable of elevating the effective intracellular concentration of cyclic GMP, so that cell-cell adhesion is disrupted.

95. The composition of claim 94, wherein said cells are brain microvascular endothelial cells and said permeability barrier is the blood-brain barrier.

96. The composition of claim 94, wherein said agent is selected from among the group consisting of an agent that elevates guanylate cyclase activity, an agent that decreases the degradation of cyclic GMP or cyclic GMP analogues or derivatives, and an agent that stimulates the entry into cells of cyclic GMP or analogues or derivatives of cyclic GMP.

97. A drug delivery composition for opening tight junctions between microvascular endothelial cells, whereby means are provided for a drug to cross the permeability barrier created by such junctions, comprising an agent capable of increasing the physiological effects of cyclic GMP, so that cell-cell adhesion is disrupted.

98. The composition of claim 97, wherein said cells are brain microvascular endothelial cells and said permeability barrier is the blood-brain barrier.

99. The composition of anyone of claims 88, 91, 94 and 97 in a pharmaceutically acceptable vehicle.

100. A drug delivery method for opening tight junctions between microvascular endothelial cells of a subject, comprising the steps of administering to said subject an agent, in an effective amount and in a pharmaceutically-acceptable vehicle, capable of reducing the effective intracellular concentration of cyclic AMP, so that cell-cell adhesion is disrupted and whereby means are provided for a drug to cross permeability barriers imposed by said tight junctions.

101. The method of claim 100, wherein said cells are brain microvascular endothelial cells and said permeability barrier is the blood-brain barrier.

102. The method of claim 100, wherein said agent is selected from among the group consisting of agents that reduce adenylate cyclase activity, agents that inhibit the functioning of G_s agonists, agents that stimulate the degradation of cyclic AMP or cyclic AMP analogues or derivatives, and agents that inhibit the penetration of cells by cyclic AMP or analogues or derivatives of cyclic AMP.

103. A drug delivery method for opening tight junctions between microvascular endothelial cells of a subject, comprising the step of administering to said subject an agent, in an effective amount and in a pharmaceutically-acceptable vehicle, capable of reducing the physiological effects of cyclic AMP, so that cell-cell adhesion is disrupted and whereby means are provided for a drug to cross permeability barriers imposed by said tight junctions.

104. The method of claim 103, wherein said cells are brain microvascular endothelial cells and said permeability barrier is the blood-brain barrier.

105. The method of claim 103, wherein said agent is an agent that inhibits cyclic AMP-activatable protein kinase or stimulates a phosphoprotein phosphatase.

106. A drug delivery method for opening tight junctions between microvascular endothelial cells of a subject, comprising the steps of administering to said subject an agent, in an effective amount and in a pharmaceutically-acceptable vehicle, capable of elevating the effective intracellular concentration of cyclic GMP, so that cell-cell adhesion is disrupted and

whereby means are provided for a drug to cross permeability barriers imposed by said tight junctions.

107. The method of claim 106, wherein said cells are brain microvascular endothelial cells and said permeability barrier is the blood-brain barrier.

108. The method of claim 106, wherein said agent is selected from among the group consisting of an agent that elevates guanylate cyclase activity, an agent that decreases the degradation of cyclic GMP or cyclic GMP analogues or derivatives, and an agent that stimulates the entry into cells of cyclic GMP or analogues or derivatives of cyclic GMP.

109. A drug delivery method for opening tight junctions between microvascular endothelial cells of a subject, comprising the steps of administering to said subject an agent, in an effective amount and in a pharmaceutically-acceptable vehicle, capable of increasing the physiological effects of cyclic GMP, so that cell-cell adhesion is disrupted and whereby means are provided for a drug to cross permeability barriers imposed by said tight junctions.

110. The method of claim 109, wherein said cells are brain microvascular endothelial cells and said permeability barrier is the blood-brain barrier.

111. A method of treating vasogenic brain edema in a subject, comprising the step of administering to said subject an agent, in an effective amount and in a pharmaceutically-acceptable vehicle, capable of increasing the physiologically-effective intracellular concentration of cyclic AMP, so that means are provided for increasing tight junctions between brain microvascular endothelial cells and so that cell tight junction permeability is reduced.

112. The method of claim 111, wherein said agent is selected from among the group consisting of agents that increase cellular adenylate cyclase activity, agents that inhibit the degradation of cellular cyclic AMP, and derivatives of cyclic AMP to which endothelial cells are permeable and which act physiologically as does cyclic AMP.

113. A method of treating vasogenic brain edema in a subject, comprising the step of administering to said subject an agent, in an effective amount and in a pharmaceutically-acceptable vehicle, capable of increasing the physiological effects of cyclic AMP, so that means are provided for increasing tight junctions between brain microvascular endothelial cells and so that cell tight junction permeability is reduced.

114. The method of claim 113, wherein said agent comprises a protein kinase stimulator or a phosphoprotein phosphatase inhibitor.

115. A composition for treating vasogenic brain edema in a subject, whereby means are provided for inhibiting tight junction permeability of brain microvascular endothelial cells, comprising an agent, in a pharmaceutically-acceptable vehicle, capable of increasing the physiologically-effective intracellular concentration of cyclic AMP.

116. The composition of claim 115, comprising agents selected from among the group consisting of agents that increase cellular adenylate cyclase activity, agents that inhibit the degradation of cellular cyclic AMP, and derivatives of cyclic AMP to which endothelial cells are permeable and which act physiologically as does cyclic AMP.

117. A composition for treating vasogenic brain edema in a subject, whereby means are provided for inhibiting tight junction permeability of brain microvascular endothelial cells, comprising an agent, in a pharmaceutically-acceptable vehicle, capable of increasing the physiological effects of cyclic AMP.

118. The composition of claim 117, wherein said agent comprises a protein kinase stimulator or a phosphoprotein phosphatase inhibitor.

119. A method for modulating leukocyte adhesion to brain endothelial cells by substantially preventing a leukocyte VLA-4 molecule from binding to a brain endothelial ~~VCAM-1 molecule~~. *ligand.*

120. The method of claim 119 wherein said method includes use of a reagent that blocks the binding of said VLA-4 molecule to said VCAM-1 molecule.

121. A method of claim 119 wherein said reagent is a receptor.

122. A method of claim 121 wherein said receptor is selected from the group consisting of an antibody, an antibody fragment thereof, and a peptide.

123. A method of claim 121 wherein said receptor is directed against a VLA-4 molecule.

124. A method of claim 123 wherein said receptor is directed against the α -1 subunit of the VLA-4 cell adhesion molecule.

125. A method of claim 121 wherein said receptor is directed against both the α -4 and β -1 subunits of the VLA-4 adhesion molecule.

126. A method of claim 121 wherein said receptor is directed against a VCAM-1 molecule.

127. A method of claim 121 wherein said receptor is a monoclonal antibody.

128. A method for substantially preventing or ameliorating brain inflammation including modulating leukocyte VLA-4 molecule adhesion to brain endothelial cell VCAM-1 molecules.

129. A method of claim 128 wherein said method includes use of a reagent which blocks the binding of said VLA-4 molecule to said VCAM-1 molecule.

130. A method of claim 129 wherein said reagent is a receptor.

131. A method of claim 130 wherein said receptor is selected from the group consisting of an antibody, an antibody fragment thereof, and a peptide.

132. A method of claim 130 wherein said receptor is directed against the VLA-4 molecule.

133. A method of claim 132 wherein said receptor is directed against the α -1 subunit of the VLA-4 molecule.

134. A method of claim 130 wherein said receptor is directed against both the α -4 and β -1 subunits of the VLA-4 molecule.

135. A method of claim 130 wherein said receptor is directed against a VCAM-1 molecule.

136. A method of claim 130 wherein said receptor is a monoclonal antibody.

137. In a method for treating chronic inflammatory brain disease, the improvement comprising administering a therapeutically effective dosage of a reagent that substantially prevents a leukocyte VLA-4 molecule from binding to a brain endothelial VCAM-1 molecule.

138. A method of claim 137 wherein said reagent is a receptor.

139. A method of claim 138 wherein said receptor is selected from the group consisting of an antibody, an antibody fragment thereof, and a peptide.

140. A method of claim 138 wherein said receptor is directed against the VLA-4 molecule.

141. A method of claim 140 wherein said receptor is directed against the α -1 subunit of the VLA-4 molecule.

142. A method of claim 138 wherein said receptor is directed against both the α -4 and β -1 subunits of the VLA-4 molecule.

143. A method of claim 138 wherein said receptor is directed against a VCAM-1 molecule.

144. A method of claim 138 wherein said receptor is a monoclonal antibody.

145. A composition for preventing or ameliorating brain inflammation including at least one reagent that substantially prevents binding of a leukocyte VLA-4 molecule to a brain endothelial VCAM-1 molecule.

146. A composition in therapeutically effective form for preventing or ameliorating brain inflammation including at least one receptor that substantially prevents binding of a leukocyte VLA-4 molecule to a brain endothelial VCAM-1 molecule.

147. A composition for treating brain inflammation including a therapeutically effective form of at least one receptor that substantially prevents binding of a leukocyte VLA-4 molecule to a brain endothelial VCAM-1 molecule.

148. A composition for treating inflammation including a therapeutically effective form of at least one receptor that substantially prevents binding of a leukocyte VLA-4 molecule to a brain endothelial VCAM-1

molecule and delivers to the brain endothelial cell an anti-inflammatory agent.

149. A method for inducing brain inflammation via the intra-cranial injection of tumor cells.

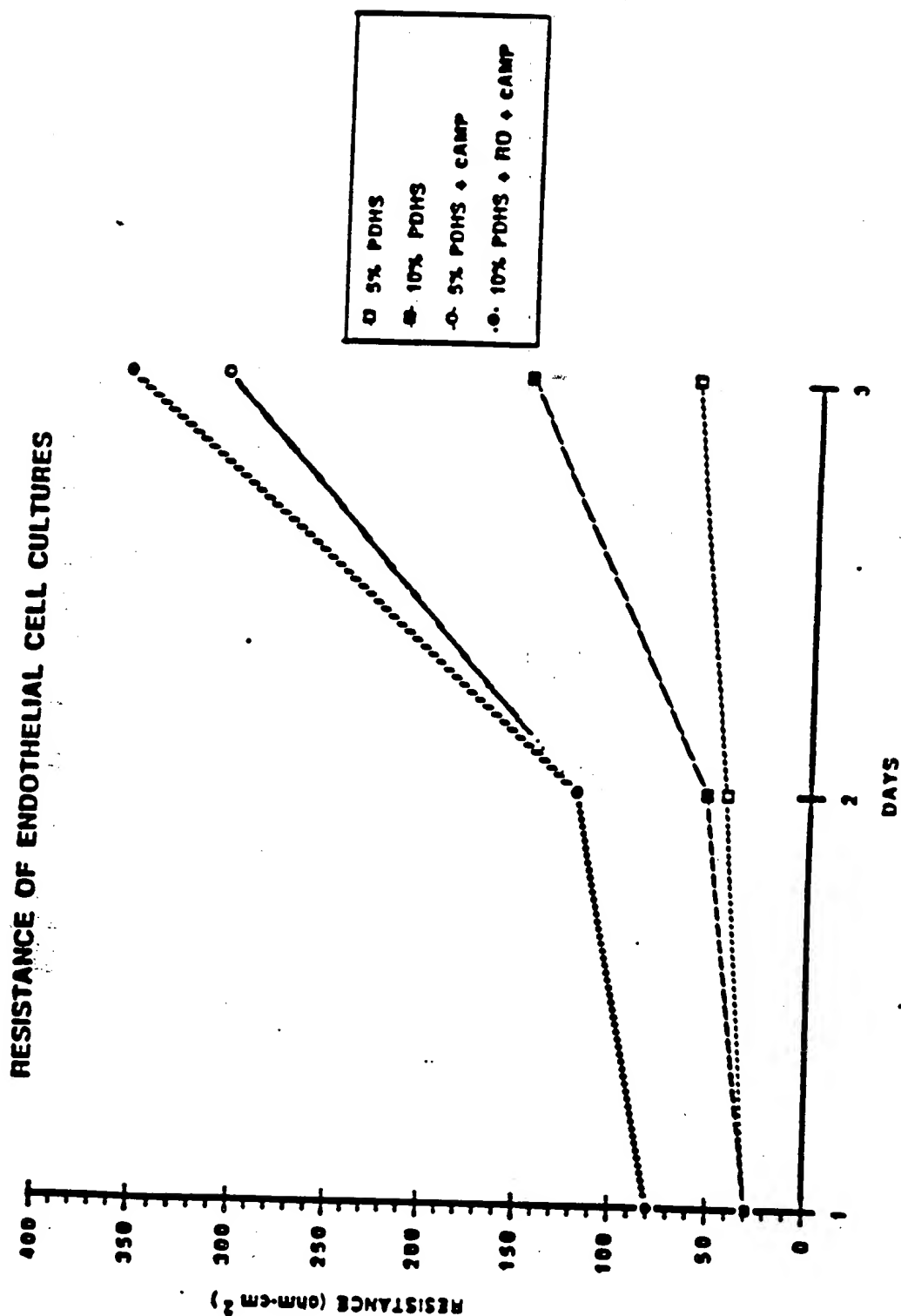
150. The method of claim 149 wherein said tumor cells are ATCC 1573 human kidney-derived.

151. The method of claim 149 wherein a rat is injected intra-cranially.

152. An assay for ascertaining whether a compound is effective as an anti-inflammatory agent in the brain, wherein said assay includes inducing brain inflammation in a living organism via the intra-cranial injection of tumor cells, exposing brain tissue from said injected living organism to said putative brain anti-inflammatory agent.

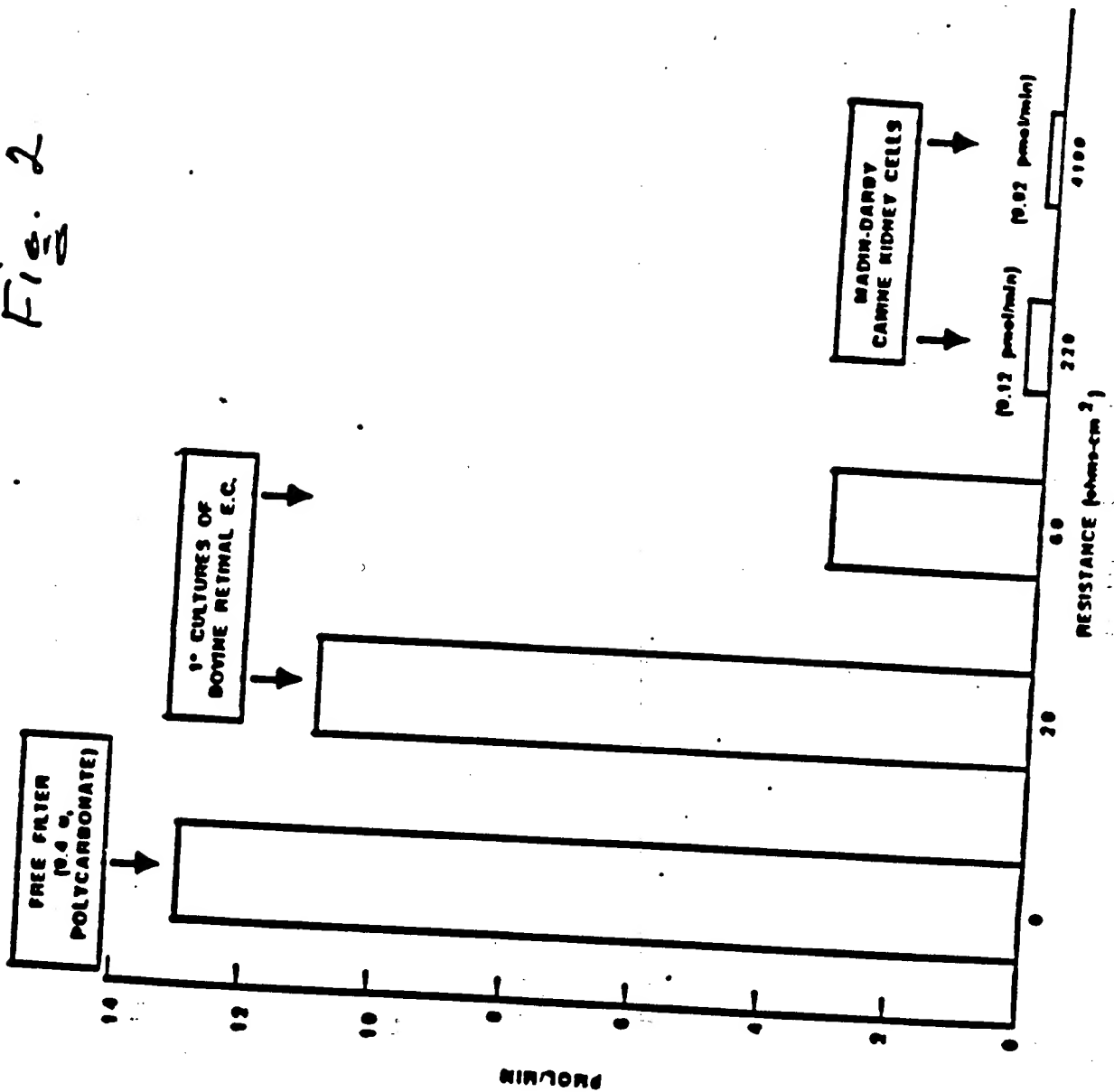
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Fig. 1



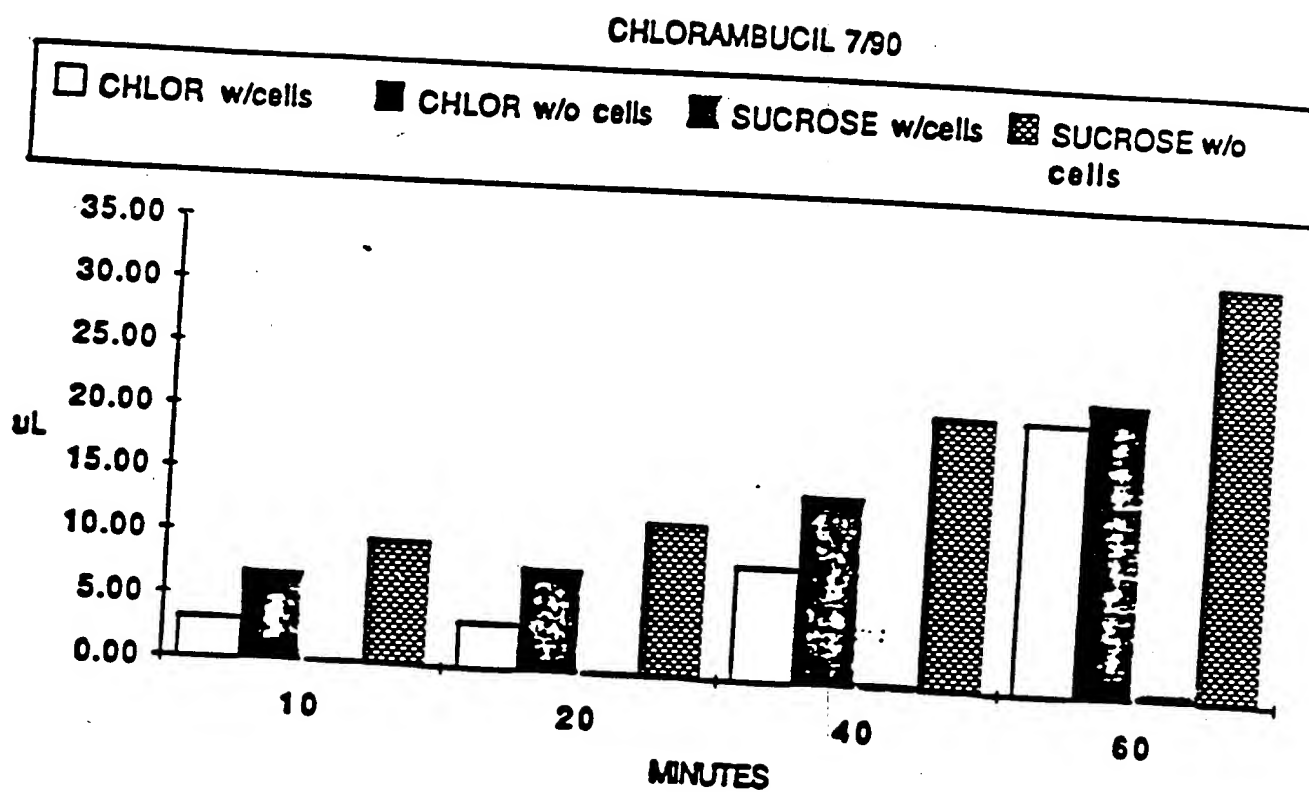
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Fig. 2



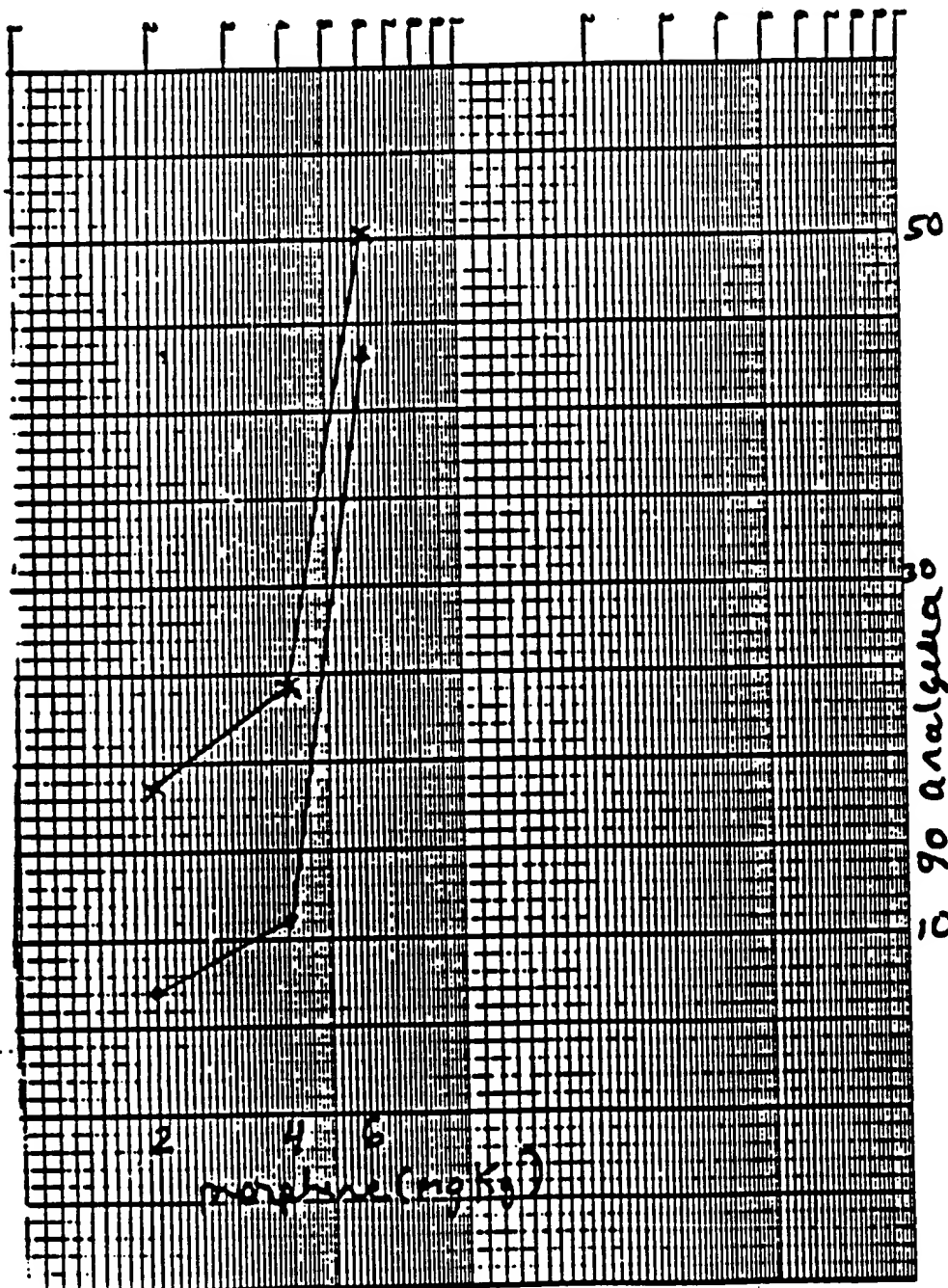
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Fig. 3



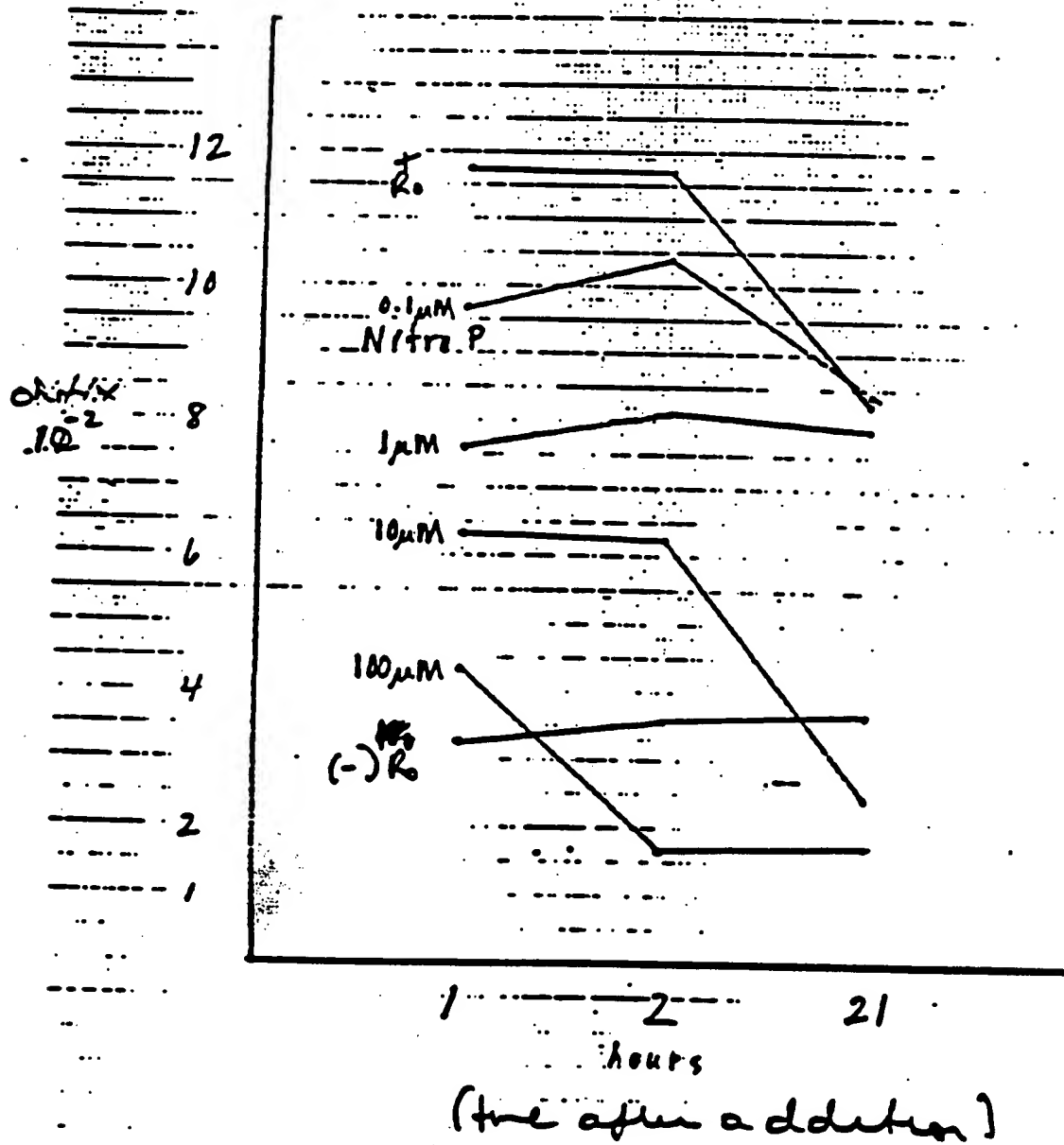
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Fig. 4

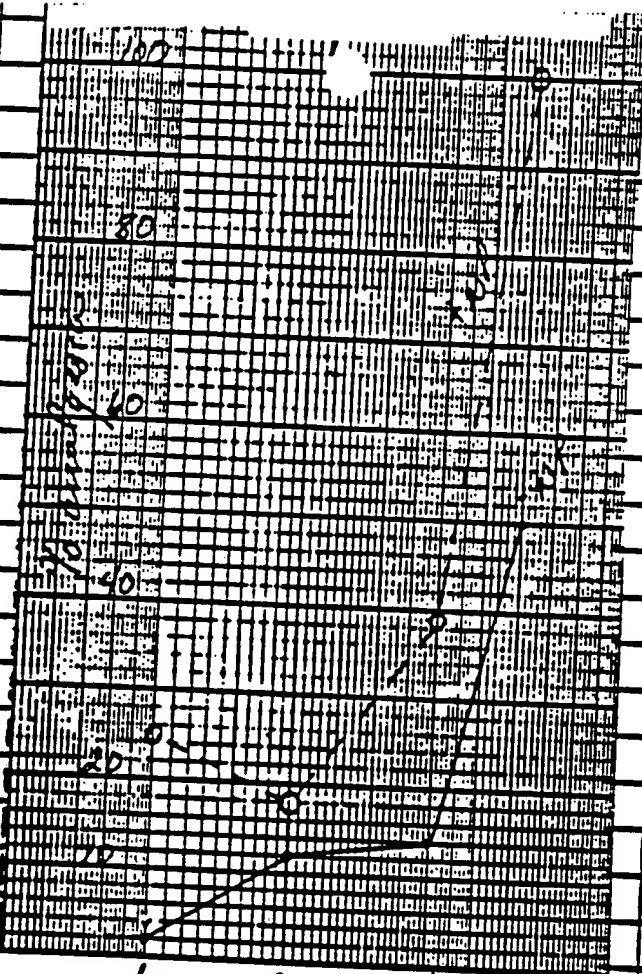


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Fig. 5



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morphine 2mg/kg 4 6

Figure 6

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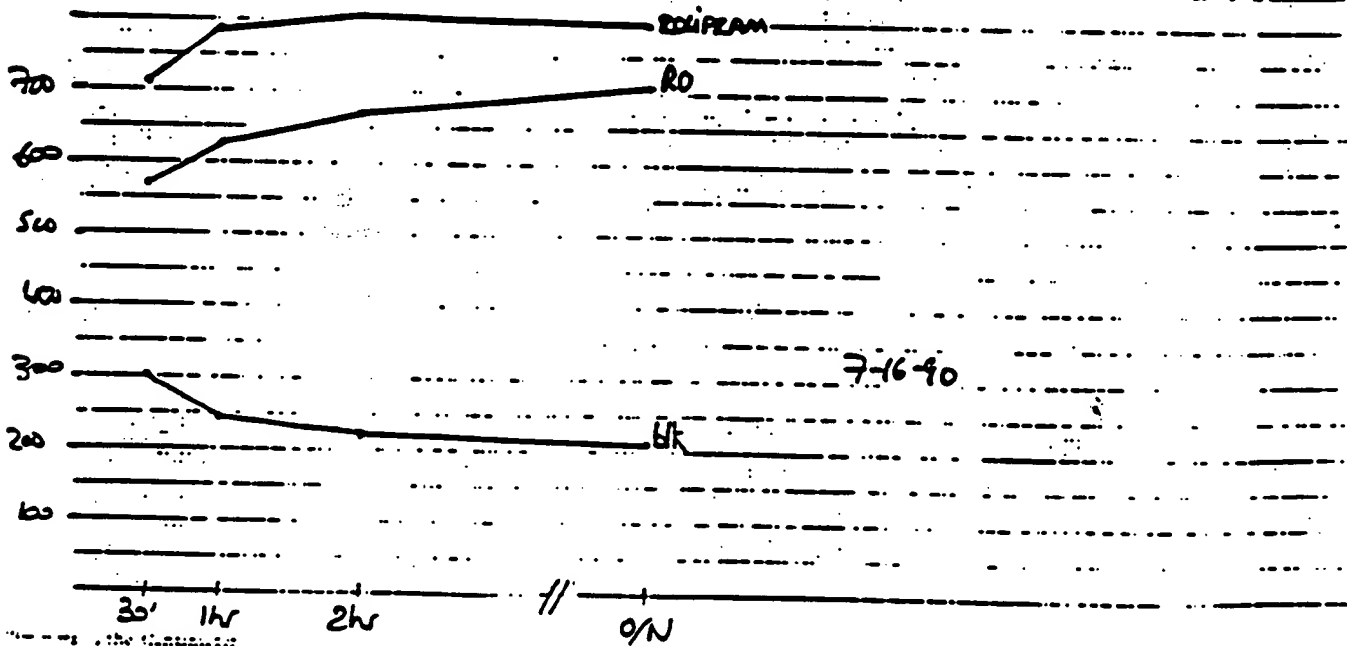
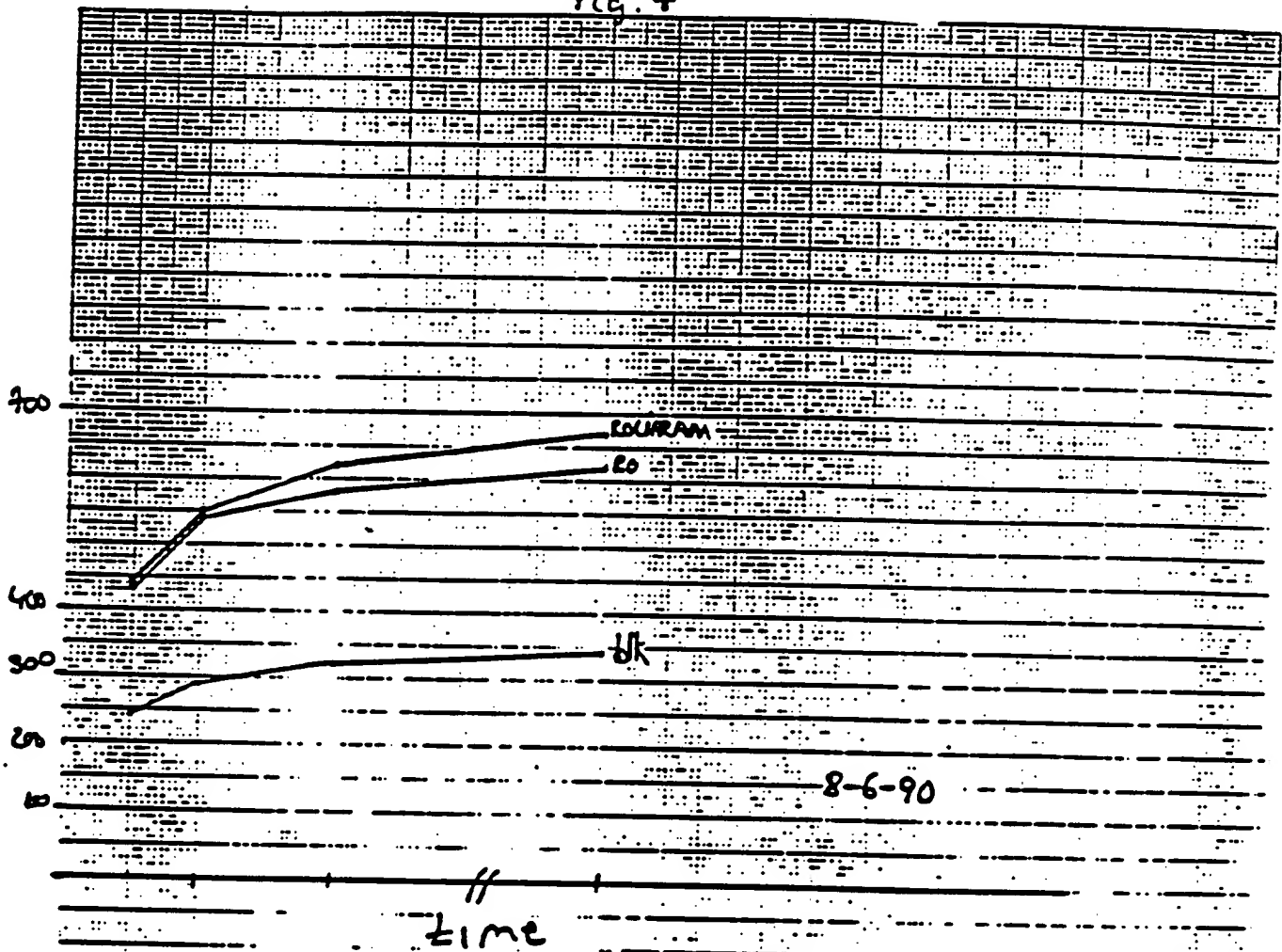


Figure 8: Brain Section Assay

A. No Treatment Binding of mixed human (large cell) and mouse lymphocyte (small cell) populations (see Methods).



B. Anti - B1 Integrin Mixed lymphocyte population as in A, treated with anti-human B1. Binding of large, human cells is inhibited.

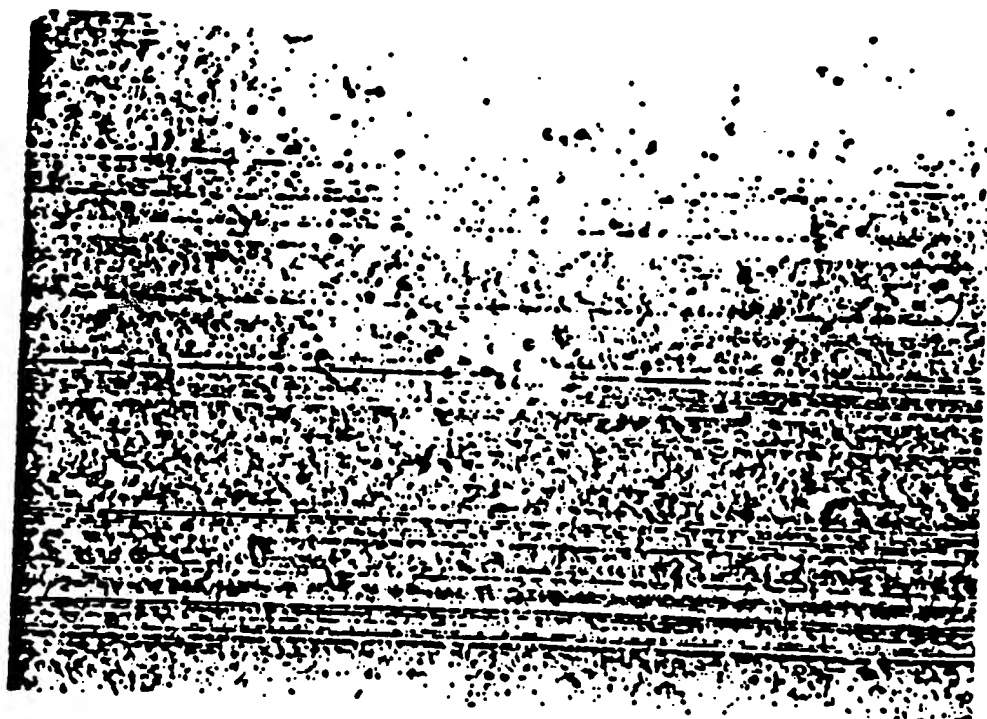
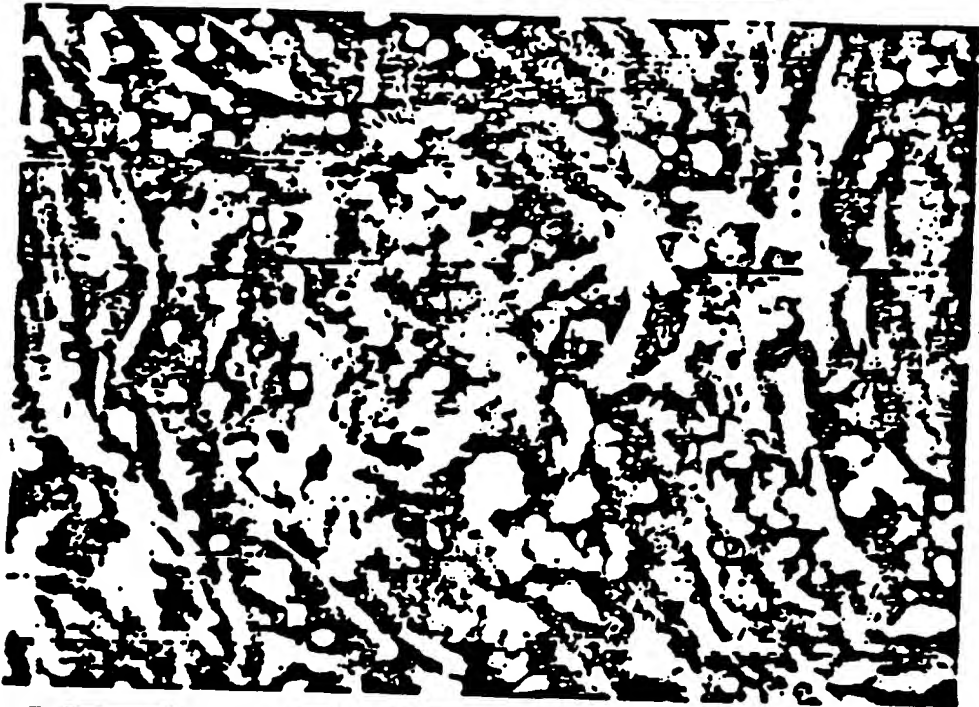


Figure 9: Cultured BBB Endothelium Assay

A. Low level binding of human lymphocytes to untreated endothelium



B. Human lymphocytes bind approximately 7-fold better to endothelium stimulated with TNF



C. Treatment of human lymphocytes with anti-B1 Integrin inhibits their augment binding to TNF-stimulated endothelium



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Figure 10. Lymphocyte Binding to Blood Vessels in Sections of Lymph Node and Inflamed Brain

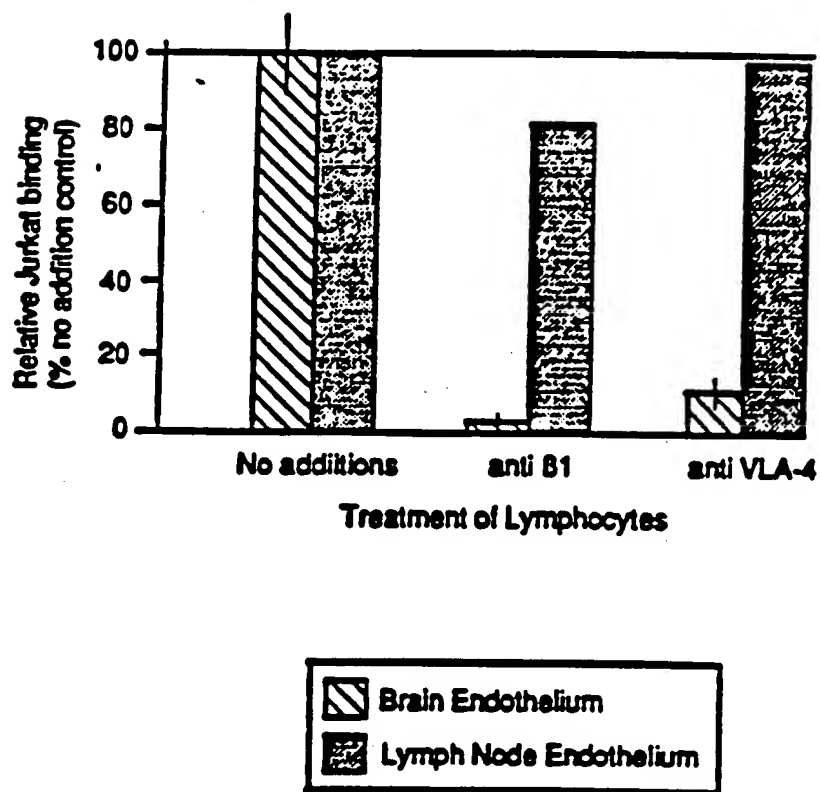


Figure 1/: Jurkat Binding to Brain EC